

CYTOLOGY
AND
CELL PHYSIOLOGY

EDITED BY
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'Cytology may rightly claim to be the frontier state in the biological commonwealth, for within its borders biologists and chemists find common ground.'

PROFESSOR JAMES GRAY, 1931

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FOREWORD

DURING an argument a Liberal and a Conservative in the old-fashioned sense discovered to their surprise that there was little in fact to choose between their idealized positions. So it is with the cytologist and biochemist; initially so widely separated in method and outlook, so far as the biochemist's concern is chemistry and life, this is also the ultimate cytological aim. Some years ago the writer of this foreword was forced to urge the need for a bolder and more imaginative co-operation from the standpoint of known biochemical facts about the nucleo-proteins, finding a possible solution of some difficulties about the living cell in a conception which has subsequently been called the 'cyto-skeleton'. Arguments borrowed from organic chemistry will do much to provide a partial explanation of the unity and division of the cell.

This book is wanted to form a permanent record of the tendency for these two previously separated groups of workers to draw together. Most people really waste the time of enforced idleness which is such an inevitable accompaniment of a war. It is greatly to the credit of the contributors here that they have fought mental lethargy to the point of creating this valuable composite statement upon the cell. The spirit is indeed hard to crush; the weakness and strength of the flesh rests upon organized chemistry.

R. A. PETERS

20 July 1941

PREFACE

THE phase of purely morphological investigation of cells is now changing into a period in which the interpretation of structure in terms of chemical composition and function is the aim of many cytologists. This does not mean that we have learnt all that the morphologist has to tell us, for there are many problems of cell structure which he has yet to solve. But it means that the morphologist will need to work, not as before in a watertight compartment, nor even in a compartment which is covered with a semi-permeable membrane, but in one which will permit an intimate mixing of his knowledge with that of the physicist, the biochemist, and the physical chemist: for so complex are cellular organization and function that the brain of no one man can hope to envisage their manifold complications.

In this book an attempt has been made to bring together chemical, physicochemical, and morphological aspects of the study of cells. It has not been the aim to cover the whole field of cytology or of cell chemistry, indeed it would take a series of volumes to do so. The best that one can do is to choose a number of subjects which are representative of different fields of the study of cells and which relate, as far as possible, one to the other, and to bring them together within a single cover.

The problems of producing such a book as this in war-time are not inconsiderable, and the editor wishes to thank the contributors, who are all scientists working in war-time Britain, for the way they overcame their many difficulties and the speed with which they produced their various chapters.

The whole book was written during the course of the 1940-1 air blitzkrieg on Britain and there are probably no chapters of which part was not written within the sound of bursting bombs. One contributor, in fact, wrote almost his entire chapter by candle-light in an air-raid shelter during the worst bombing attacks on London. Another author wrote his while on sick leave from one of the fighting services, and a third produced his contribution chiefly in railway carriages while travelling from one urgent war duty to another. Most authors have written their chapters in what little time they had left over from war research. Others who have not been occupied directly in this way have had their time severely curtailed by other war duties such as acting as air-raid wardens, &c.

Incidental difficulties have harassed the authors in various ways. Those who were working in areas subject to frequent air attack had to contend with the closing of libraries while air raids were in progress, or with the evacuation of libraries to other parts of the country. The

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latter difficulty often resulted in long and tedious journeys to obtain essential periodicals.

Thanks are also due to Professor R. A. Peters for his interest in the subject of this book and for sparing time from his many urgent duties to write a foreword for it.

It is a pleasure to acknowledge the care and attention which the staff of the Oxford University Press have given to the production of the book and to congratulate them on the speed with which, despite their occupation with urgent war work, they were able to place it in print.

G. B.

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CHAPTER I

SOME ASPECTS OF CYTOLOGICAL TECHNIQUE

By JOHN R. BAKER

i. INTRODUCTION

THANKS to recent advances in histochemistry, it is nowadays once more possible for the study of substance to go hand in hand with the study of the minute structure of organisms, as they went long ago when the introduction of histological staining had not yet driven a subtle wedge between the two. To-day the cytologist is or must become something of a biochemist, but a biochemist of an unusual sort. It was once remarked to the writer that biochemists like to have their substances in test-tubes. The cytologist wants to have his exactly where they were in life, and to know, as precisely as he can, what they are. When substance and structure are known, the way is clear for the elucidation of the main problem of cytology, which is to discover what a cell does to keep alive and to perform its functions for the body as a whole or for the next generation.

To some extent cytological technique is a craft. No one will succeed in it who finds its processes tedious. To ensure the making of a good cytological preparation, of whatever kind, it is necessary not only to take such precautions as have been proved to be valuable in the particular case in hand, but also to do everything that may reasonably be supposed to help towards the desired end. Often there are many small ways in which one can prevent distortion of cellular detail, and it is not possible to prove conclusively that each one is actually helpful in every case; but if all the small precautions are omitted for the sake of speed or as a result of laziness, success is unlikely. The man who regards technique almost as a craft and will take endless trouble to approach perfection in his work is the one who is likely to increase knowledge of cellular structure and substance.

Time may often be saved and success secured by the adoption of long techniques. In these days when rush-methods are appreciated beyond their worth, many people are prepared to waste almost any amount of time if only they can get results quickly. The quick methods often take up more of one's time than the slow. The leaving of a slide overnight in a solution occupies none of anyone's time: the holding of it under the same solution over a flame engages some one's close attention for several minutes, and delicate structures may be injured by the heat or consequent precipitation.

It must be allowed that some procedures are unnecessarily tedious. The profusion of different substances used and the complication of many of the methods often seem out of all proportion to the value of the results obtained. It is a striking experience to make a permanent preparation of a mammalian testis by Benda's hugely elaborate process—involving, as it does, prolonged fixation followed by two separate mordanting processes of the tissue in bulk and two further mordanting processes applied to the sections, and then staining of the mitochondria and a very tricky differentiation, and lastly dehydration mainly by filter-paper and passage into balsam via bergamot oil—and afterwards to tease up a piece of fresh testis in saline and see the mitochondria without any fixing or staining process, transparent indeed and uncoloured, but as clear to a sensitive eye as when intensely stained violet in the permanent preparation.

ii. FRESH MATERIAL

When cells fall apart readily—and unfortunately they generally do not—fresh teased preparations in indifferent media are usually preferable to stained sections. In this simple way the Golgi complex was studied by Platner⁶⁰ in 1885 in fresh spermatocytes and spermatids of the snail. His figures, though not exact, give a good impression of what the living cell is actually like; yet so accustomed are people to the caricature of cellular structure produced by reagents that when the writer exhibited one of Platner's figures at a scientific meeting it was greeted with laughter.

The transparency of fresh preparations is so great that the use of certain dyes of small toxicity is usually very helpful. Many basic dyes are suitable, perhaps as suitable as the neutral red and Janus green which have gained such wide acceptance. It is desirable, where possible, to have the cells suspended in a body fluid of the animal from which they were taken; and when it is proposed to do this, it is best to dissolve the dye or dyes in absolute alcohol and precipitate them on glass microscopical slides by evaporation of the alcohol.⁶⁷ When subsequently the body-fluid containing the cells is added to the slide, the dye dissolves and acts on the cells without any other external influence (whether of osmotic pressure or of salt balance) being present to interfere.

Acid dyes cannot effectively be used supravitality in this way, because they do not then tend to show pre-existing structures; but some of them can most profitably be injected into living animals, certain of whose cells will take them up in a characteristic way.¹³ The dyes which are useful in this respect are chiefly acid disazo dyes, with molecules so large that the solutions may be regarded as colloidal. On account of its low toxicity, high intensity of staining, and resistance to subsequent treatment, trypan blue is probably the best of these. When the cells

have had time to take up the dye, the animal is killed and the tissue cut out. The cells may then in certain cases be examined directly, without further treatment, but it is generally convenient to preserve the tissues in an ordinary fixative and cut paraffin sections, which may be further stained if it is desired. The resistance of the dye to extraction by the solutions in which the tissue is subsequently soaked and in which it is perfectly soluble is very surprising, and must be attributed to the particular physical form in which the enclosing proteins are precipitated by the fixative. Of the dyes other than disazo compounds which may be used intravitaly in this way, isamine blue is the most noteworthy. This acid dye belongs to the triphenylmethane series, and once again has a very large molecule.

The cells of the reticulo-endothelial system have a strong tendency to take up and retain the large molecules of these vital dyes, while failing to absorb or retain, as the case may be, both basic dyes and such acid ones as have small (or extremely large) molecules. These cells are phagocytic, and the taking-up and retention of the vital dyes may be regarded as a process in which they are actively concerned; further, the vacuoles in which the dyes are seen to accumulate may be considered as *sui generis* and not corresponding to any cell element of general occurrence. It is otherwise with the cells of the convoluted tubules of the vertebrate kidney, and here a question of major interest is whether the vacuoles revealed by the dye are to be regarded as having any relation with the vacuolar component of the Golgi complex.

The fatal facility of the microtome has made investigators nearly always study cells in sections if it is decided to study them dead. Cytologists will often describe in minutest detail the shape of every structure contained within a cell, while leaving the shape of the cell itself unmentioned or represented by drawings that give no adequate concept. It was not always so. Before the days of microtomy, histologists were expert at the separation of cells by maceration. In all probability few present-day cytologists have tried iodized amniotic fluid as a macerating agent, but in the writer's experience this 76-year-old method⁶⁹ is a useful one. Ranvier's remarks⁶² on the techniques of maceration and mechanical dissociation, written in 1875, are well worth study. The early histologists could not only demonstrate relatively gross structure (such as the course of the vertebrate uriniferous tubules) in a way that would be startling in its demonstrativeness to the section-minded student of to-day, but also knew much of intimate cell structure that passes unnoticed when the cell has been through the mill of present-day cytological technique. It is interesting and instructive to turn over the pages of Beale's *How to work with the Microscope*⁹ and to marvel at how much was known before the invention of rocking and rotating microtomes. What is particularly wanted at the present day is a

macerating fluid which will preserve the mitochondria and Golgi complex in a lifelike form. Professor E. S. Goodrich has recently found that a saturated aqueous solution of boric acid is a valuable macerating agent for many tissues. His account will appear in the *Quarterly Journal of Microscopical Science*.

iii. FIXED MATERIAL

a. Fixation.

Nowhere in cytological technique has empiricism run riot so freely as in the invention of fixative mixtures. Substances which will obviously react together are mixed haphazard (as, e.g., when chromic acid is added to Bouin's fluid¹) and no one can say to what extent the tissues are affected by the original constituents and to what extent by the products of their reaction. Even when no such extra complication exists, the chemistry and physics of fixation are excessively difficult, and it is improbable that the mind of man will ever be able to elucidate just what happens when a piece of tissue is placed, for instance, in Bouin's fluid. The speed at which each constituent penetrates, the way in which each acts singly upon each of the different substances present in the cells, how one constituent affects the other two as regards degree of dissociation and chemical action, these are some of the problems awaiting solution even when so simple and rational a fixative as Bouin's is employed.

The histologist calls a fixative 'good' when the tissues are evenly and slightly shrunken and the nuclei stand out sharply in the stained section, but the sharpness of the precipitated chromatin is no evidence of lifelike preservation, and the only evidence of what is good or bad is comparison with the living cell. This point cannot be too strongly stressed: subjective ideas of what final result is good and what bad should never be allowed to form in the mind except on the solid ground of comparison with what is visible in the cell while still unaffected by reagents. Fry²⁷ has insisted that one should not choose for careful study what one thinks to be the 'best-fixed' cells in a preparation, but should pay equal attention to a number of cells chosen at random. This is true, unless certain cells show a particularly close resemblance to what one knows to be their structure in life.

From the welter of different fixative mixtures it is difficult to extract any clear principles for the understanding of why one gives lifelike preservation while another does not. One fact may, however, be pointed out: most reliable fixatives contain one or more substances which precipitate the proteins of the cytoplasm, and one or more which render those proteins insoluble without precipitating them. This applies to fluids so valuable to the cytologist as Flemming's, La Cour's, Aoyama's, Sanfelice's, Helly's, Mann's, and Bouin's. The non-precipitant fixatives in these mixtures are osmium tetroxide, unacidified potas-

sium dichromate, or formaldehyde. One might fancy that non-precipitant fixatives used alone would give the best results, since precipitation causes the formation of artificial structures; but in practice it is found that precipitated proteins are more easily penetrated by the substances used in subsequent treatment, particularly paraffin, and more easily stained. Thus Altmann's fluid (osmium tetroxide and potassium dichromate), excellent in its way, gives an inconveniently crumbly consistency to tissues, and Regaud's (formaldehyde and potassium dichromate) fails badly with certain tissues (e.g. the spermatogenic tissues of many animals). The omission of the non-precipitant fixatives, however, as in Zenker's fluid, results in precipitation of the cytoplasm in a meshwork whose coarseness causes artificial appearances which are serious for the cytologist, though not necessarily for the histologist. (It will be recollected that the potassium dichromate in Zenker's fluid, being in an acid medium, is a precipitant fixative.⁷⁸)

It seems strange that the addition of potassium dichromate—a solvent of nucleoproteins except in rather strongly acid solution—can improve the action of a chromosome fixative, but La Cour's fluids contain it.³⁷ The reason for the success of these fluids is perhaps to be attributed partly to the even fixation of the proteins which lie round the chromosomes, and to this even fixation the dichromate contributes. Chromic acid, which is also present, ensures that the nucleoproteins are not dissolved. It is clear that violent protein precipitation in the immediate vicinity of a chromosome would distort its structure, more particularly when that structure is especially fragile (e.g. in the early diplotene stage of gametogenesis, when the presence of four strands is often obscured except by the employment of delicate techniques).

The mixing of formaldehyde with oxidizers may perhaps be excused on the practical ground that it is done in some very good fixatives, notably in Helly's fluid and those Flemming variants in which formaldehyde is substituted for osmium tetroxide. The writer's fortune in having seen some of Dr. M. J. D. White's beautiful chromosome preparations made with Sanfelice's fluid almost reconciles him to the use of unstable mixtures. The reaction of potassium dichromate or chromic acid with formaldehyde is rather a slow one, and it may be argued in favour of such mixtures that for a considerable time a part of the oxidizer and a part of the reducer are present and exerting their effects on the cells concurrently, the reaction products being of no consequence. This may be so, but it would be worth someone's while to find out just what the reaction products are in the case of a few valuable unstable fixatives (e.g. Helly's³⁰ and Sanfelice's³⁶), and what effects, if any, they have on cells. Helly seems to have had a feeling that the effects of the reaction products might be damaging, for he recommends that if fixation lasts for more than 6 hours the tissue should be removed from his unstable

mixture and transferred to a fluid containing the other constituents but not the formaldehyde.

Until the effects of the reaction products have been investigated, one cannot help being harassed by such thoughts as these. If it is desirable that the dichromate or chromic acid should oxidize something in the cells, why is formaldehyde added, which will interfere with the oxidation; and vice versa, if reduction by formaldehyde is desirable, why add dichromate or chromic acid? Or is one seeking only those effects of these substances that involve neither oxidation nor reduction? The truth is that when one uses these unstable mixtures one knows even less about what one is doing than when a stable mixture is used (which is saying a good deal).

The question whether salts devoid of fixing properties should be added to fixatives to prevent changes due to osmotic pressure is still very obscure. For some unexplained reason formaldehyde has long been the fixative to which it is deemed especially desirable to add sodium chloride for this purpose, though it is many years since Meves⁵¹ treated chromic acid and mixtures made with it in the same way. It has been shown⁵ that many fixing mixtures are hypertonic, some enormously so, and it is not immediately obvious how the addition of sodium chloride or other salts to them could prevent osmotic changes in the cells. However, Carleton¹⁴ and Young⁷⁷ have found that with certain fixatives and certain tissues there is a demonstrable advantage in adding an indifferent salt. In the absence of the added salt the cells are shrunken. Young supposes that when a piece of tissue is placed in a fixative solution the ingredients of which are slow in diffusion and to which no indifferent salts are added, there is a loss of ions from the intercellular fluids in the unfixed centre of the piece, and hence an osmotic swelling of the cells in the vicinity. He supposes this swelling to go so far as to result in bursting, and he thinks that later the fixative molecules arrive and act upon the already burst—and then shrunken—cells. This hypothesis cannot be accepted until it has been shown that the swelling and bursting, followed by rather inexplicable shrinkage, do actually occur. One must keep in mind also the excellent results often obtained when fixatives are made up without added indifferent salts. For the present one may decide to add salts (or dissolve in sea-water) when using a slowly diffusing fixative with marine invertebrate tissues. Judgement may be suspended, however, both as to the *raison d'être* of the procedure and as to its desirability, as a general rule, with the cells of vertebrates and terrestrial invertebrates.

The addition to fixatives of substances which reduce surface tension seems likely to be helpful, and it may be suggested that it would be profitable to try hexylresorcinol in this way. Ordinary soaps do not commend themselves on account of their alkalinity, but esters of tri-

ethanolamine might prove valuable. La Cour³⁷ has already put saponine in his fixatives for chromosomes.

Despite the invention of some useful fixatives in recent years, it remains true that to the present day one of the most valuable is Flemming's fluid (strong formula), first published in 1884,²⁶ which is still among the best both in the intricacies of modern chromosome studies and for the demonstration of mitochondria. For the latter purpose the acetic acid is reduced below 5 per cent., and it is to be remembered that Flemming himself suggested that figure as the maximum to which the acetic acid should rise, and not the standard figure for all purposes.

A technique which seems to have been overlooked is Overton's old method⁵⁴ of removing the excess of chromic acid and potassium dichromate from tissues without prolonged washing in water. This consists in giving the tissues a relatively short bath in sulphurous acid solution. (The same solution as is used in Feulgen's technique is convenient.⁷) It would appear that the chromium left in the tissues is converted to $\text{Cr}_2(\text{SO}_4)_3$, which acts as a mordant to subsequent staining with such acid dyes as haematein and carmine; and this lessens the difficulty of staining with the latter dye when chromium is present in the fixative. The method does not, however, render the tissues easily stained when osmium has been a constituent of the fixative in addition to chromium.

b. Embedding.

In the embedding of tissues in wax it is probable that scarcely enough use has been made of paraffins of low melting-point. Ligroin (otherwise known as benzine or petroleum spirit and scarcely differing from certain aviation petrols) consists mostly of hexanes and heptanes and has a boiling-point near to that of water, while the so-called petroleum ether, consisting mostly of *n*-hexane, is a mobile and volatile fluid boiling at about 50°C. Both are miscible with absolute alcohol, and therefore provide valuable stepping-stones between the latter and paraffin wax. The hardening effect of the ordinary dealcoholizing agents on certain tissues can thus be avoided. Cedarwood oil is, of course, noted for its failure to harden, but it is rather expensive and messy in use. Benzene is probably the best of the ordinary dealcoholizing agents. A very convenient intermediary between this and melted hard paraffin is a mixture in equal volumes of benzene and medicinal paraffin, which has been in routine use by the writer for many years. The mixture is used cold, and no injury to tissues results from long immersion in it. The mixture may be used repeatedly.

When for any reason it is difficult or impossible to embed at once in hard wax, tissues may be stored in liquid medicinal paraffin and embedded later.⁷ This method will be found especially convenient on scientific expeditions, when a paraffin oven is not available.

For a reason which is not apparent, the embedding of tissues in paraffin wax affects the capacity to take up dyes. A piece of tissue which will accept carmine readily in bulk may resist the same dye when cut into paraffin sections. Celloidin embedding does not appear to have the same effect.

It has often been assumed that while tissues are embedded in paraffin, they are impermeable to water, but this is far from being true. Material fixed in picric acid often gives a yellow colour to the warm water used for flattening the sections, and conversely it is possible, as Walsem⁷⁴ and others (e.g.⁴⁹) have shown, to stain sections with aqueous solutions without removing the wax. The writer's own students have tried to do the same thing, admittedly with less success and through nervousness rather than of set purpose, during the course of practical examinations. The method has been used in certain cases in which treatment with alcohol impairs the action of the dye, as in the metachromatic staining of mucin. Paraffin blocks are sometimes soaked in water to make subsequent cutting easier, and it has been thought that the effect of the water is simply to cool the wax. This is not so, for the method does not work unless the tissue is first exposed at the surface of the block. Water by itself, however, is not the most desirable fluid in which to soak paraffin-embedded tissues to soften them, and a special aqueous reagent has been designed for the purpose,⁶ which penetrates paraffin-embedded tissues more readily than water and softens them better. The British Drug Houses, Ltd., are marketing a reagent of this kind.

Water is in certain cases damaging to tissues contained in paraffin. To get good preservation of the shape of mucin granules, Bizzozzero long ago¹² avoided flattening paraffin sections on water, and instead removed the paraffin with turpentine and passed them into alcohol before bringing them on to a microscopical slide and exposing them to aqueous media. Leach³⁹ found it best for certain purposes to flatten sections on 96 per cent. alcohol instead of water, and later Carleton and he¹⁵ discovered that glyceryl diacetate is a still better fluid on which to flatten. This substance is a protein precipitant, and the authors think that it works by fixing certain proteins which have avoided being acted upon by the original fixative on account of their having been bound to lipides, from which they are set free subsequently by the solvent action of the dealcoholizing fluid. The method is troublesome, but appears to merit the extra effort in particular cases.

Cytologists usually prefer to embed in paraffin, but collodion (celloidin) sometimes offers advantages. Very thin sections are not obtainable, but the smaller amount of shrinkage to some extent compensates for this; for the more the cells shrink, the thinner must be the sections if one is to avoid the occlusion of one cell by another. When serial sections are not necessary—and often in cytology they are not—collodion work

takes up little if any more of one's time than paraffin, though the final slide is not obtained so soon. The spaces between cells are probably more faithfully preserved than when paraffin is used, and also it is useful to have a sort of control against the production of artificial appearances by the hot paraffin.

It would be a convenience if manufacturers of materials for use in microscopical technique would provide two or three ready-made solutions of celloidin in ether-alcohol, each standardized by viscosity and not by the percentage of celloidin in it. Celloidin is not a single chemical entity, but a mixture in variable proportions of the trinitrate and tetranitrates of cellulose. An 8 per cent. solution, so often recommended for definitive embedding, may be either too thick or too thin, according to the particular mixture of nitrates used. When a solution of standardized viscosity is used, one can get blocks of the same hardness every time by always evaporating to the same degree; and this is easily achieved if the evaporation is always done in the same or similar capsules or dishes.

c. Staining.

Certainty does not seem yet to be approached in the old problem of whether cytological stains must be regarded as acting physically or chemically. There is much that attracts in the chemical theory, but it gives one pause when one notices—as every cytologist must have noticed—that it is the basic dyes that cling so annoyingly to glassware, while the acid ones are readily removed by water.

Lack of understanding of the action of dyes is heightened when one does not even know what dye one is using. Cytologists would do well to ask for dyes by their chemical formulae or numbers in the *Colour Index*,⁶⁶ rather than by fancy names, which often stand for variable mixtures. Conn¹⁹ has done a service in pointing out that such dyes as gentian violet and the methyl violets, which are mixtures in various proportions of hexa-methyl pararosaniline with the penta-methyl and lower compounds, are less desirable than the bluest of the lot, namely the pure hexa-methyl compound (crystal violet).

When a mixture (deliberate or fortuitous) has been long on the market under a single name, and its properties with tissues have become known, a manufacturer who produces the single substance which should rightly be known by that name may be criticized by workers for not producing the proper dye. This is not as it should be. The manufacturer who sells a mixture as a single substance deceives the worker and impedes scientific discovery. Where mixtures are beneficial, the cytologist using them should know the ingredients. One has heard it said that the indulin of one manufacturer is 'good', of another 'bad'. The words are inapplicable. The question is which manufacturer can pro-

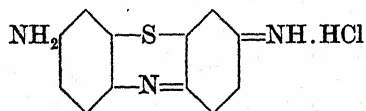
duce the purest specimen of that particular indulin, of known composition, which has a desired effect.

Cytologists would serve the interest of their science if they would restrict their purchases of materials to chemically identified products, instead of using unknown reagents with fanciful trade names. If cytological technique is to be a scientific subject, the reagents used must be known; and it would be desirable if chemical manufacturers could be induced to compete in the purity of their products rather than in the supply of media of unstated composition, whether for staining or for embedding or mounting or any other cytological purpose.

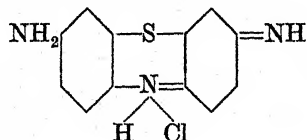
In staining, as in other branches of technique, practice often long precedes theory. When it became known that compounds between methylene blue and eosin presented great advantages in the staining of blood, it was found that the nuclei of parasitic Protozoa in such preparations were stained reddish and not in the same purple colour as the leucocyte nuclei. Since the cytoplasm of the parasites was stained blue, they showed up very distinctly in blood preparations. The complications behind these staining reactions are enormous. The methylene blue and its derivatives are present in the blood-stains partly as pure substances and partly in combination with eosin. Giemsa showed that methylene blue and eosin alone did not account for the red staining of the Protozoan nucleus, and that it depended on the appearance in the dye of a new substance, not added as such, namely methylene azure, which has one or two of the methyl groups of methylene blue replaced by hydrogen. He produced the justly celebrated stain which bears his name²⁸ by using a definite quantity of methylene azure as well as methylene blue. It was left to MacNeal to show that even methylene azure is not the direct cause of the desirable red staining of the Protozoan nucleus. Of the two azures, the one with two methyl groups (asymmetric dimethyl thionine), instead of the four in methylene blue, seems to be the more significant in this respect, though evidence has recently been produced⁶³ that the trimethyl dye is also helpful. MacNeal showed⁴⁶ that the azure acts by transformation to yet another substance, namely, the methylene violet of Bernthsen. He therefore included Bernthsen's violet in his well-known tetra-chrome stain. The complications, however, do not end here, for neither asymmetric dimethyl thionine nor its derivative, Bernthsen's violet, alone stains Protozoan nuclei reddish: a mordant is required. Now resorcinol and a few other substances will act as mordants for this particular staining reaction, and one of the other substances is eosin, itself a resorcinol derivative. Thus the eosin of methylene-blue-eosin blood-stains is in this respect acting as a mordant and not a dye.³⁸ As a last complication, the granules of mast-cells are stained a reddish colour metachromatically by the methylene blue derivatives, without the intervention of a mordant.⁵⁶

These scarcely credible complications cannot have been dreamt of by Romanowsky⁶⁴ when first he put methylene blue and eosin together in equimolecular proportions and noticed the reddish coloration given to the nucleus of the malarial parasite, never till then differentially stained. In 1901 Leishman produced his simple but excellent stain,⁴⁰ in which the valuable methylene blue derivatives were produced by the action of sodium carbonate on the parent dye. To-day the proportions of the derivatives are generally carefully controlled in blood-stains, but Leishman's product still finds a place in modern technique.

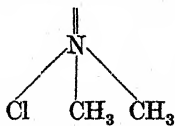
A sharp distinction must be drawn between the polychrome effects produced by the methylene blue blood dyes on the one hand, and metachromatic staining on the other. In the former, several different chemical entities are present, either as a result of the spontaneous production of chemically different substances or because such substances were deliberately added. In metachromasy there is no addition or subtraction of atoms, but only a rearrangement of the atoms within the molecule. The thiazine dyes are on the whole particularly metachromatic, and one may follow Michaelis⁵² in believing that thionine is to be represented as



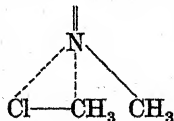
when it is staining chromatin blue, and as



when it is staining mast-cell granules or mucin or the matrix of cartilage red (see p. 27). With other groups of dyestuffs it is less easy to suggest a possible tautomeric change. Holmes³³ thinks that while the accepted



group exists in the triphenylmethane dye crystal violet when it is staining blue, this changes to



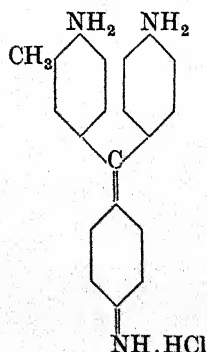
when it is staining red. In the latter case the nitrogen is really trivalent, and the methyl chloride is only held to it by 'residual affinity' Mere

dilution of a concentrated aqueous solution of crystal violet changes the colour from reddish to blue.

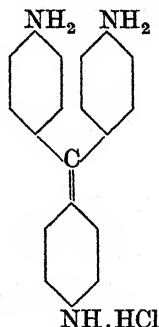
The fact that acidity favours the action of acid dyes and alkalinity that of basic ones has fostered the idea that stains should be dissolved in buffered solutions at definite pH. Levine's careful work⁴³ has shown that pH is not the only factor that matters: different buffer solutions at the same pH, and the same buffer solution at the same pH but different concentrations, have different effects on the intensity of staining. It does not seem important generally to control the pH of staining solutions at all carefully, except perhaps the methylene-blue-eosin blood-stains. It is desirable to dilute Giemsa's solution with 0.005 per cent. sodium bicarbonate solution, instead of distilled or tap-water. The action of the basic dyes in the solution is accentuated.

In the literature of staining methods one finds much that is difficult to comprehend. For instance, Mallory stated⁴⁷ that the effect of the oxalic acid in his well-known connective tissue method is to make the aniline blue stain more quickly and intensely, while the phosphomolybdic acid slows the action of the aniline blue and prevents it from gradually staining everything. It seems difficult to understand how this simultaneous application of the accelerator and brake could be the most advantageous means of achieving the desired end, though it may be so. Again, in the same technique an unmordanted acid dye (acid fuchsin) is used to stain the chromatin. Although this can be done, yet it cannot always be done effectively and with certainty; and this is the weak point in a very valuable method, in some of whose successors the fault has been corrected.

For the study of chromosomes no dye is more used to-day than gentian violet. As was mentioned before, it is probably always better to use crystal violet than that uncertain mixture of violet dyes to which the name gentian is applied. The method is essentially that introduced into bacteriological technique by Gram. This depends upon the fact that certain basic dyes react in a special way with iodine. While rosaniline



and related dyes do not react with iodine, pararosaniline



and other triphenylmethane dyes resembling it in lacking the methyl group (e.g. crystal, gentian, and methyl violets) appear to be capable of forming compounds which may be called iodo-pararosanilines.³⁸ These substances have a blue-black colour, different from the violet of the untreated dyes. In Gram's method and its derivatives, the tissue is first stained in the violet dye and then treated with iodine-iodide solution. Certain objects, such as Gram-positive bacteria and chromatin, retard the extraction of the iodo-pararosaniline compound by alcohol and other solvents; others let it go freely. Differentiation in alcohol or in alcohol followed by clove oil produces the desired result.

Hermann in 1889³¹ took this method from bacteriological technique and used it for staining chromatin in amphibian and mammalian spermatogenesis. He used an aqueous iodine-iodide solution, as did Gram. The modern technique, which has proved so valuable to those working on chromosomes, was introduced by Newton,⁵³ and further particulars have been given by Huskins³⁴ and La Cour.³⁷ The great advantage of the technique are the glassy transparency of the cytoplasm and the even action of the dye over the whole depth of the section, even when it is as much as 40 μ thick. The finest details of chromosome structure are revealed. The rather inexplicable part of the technique is the use of an alcoholic iodine solution and the use of potassium iodide in it. With aqueous solutions the iodide is necessary to dissolve the iodine, but the latter is readily soluble in 80 per cent. alcohol. Just what happens in the very short alcoholic iodine bath is obscure. The alcohol must be tending to extract the dye as such, while at the same time the iodine must be tending to convert it into the less readily removed iodo-pararosaniline derivative. Whatever actually occurs, nothing remains that shows much further resistance to alcohol, for only a few seconds must be allowed in absolute alcohol before the more leisurely differentiating action of clove oil is brought to bear. The writer would favour a fairly prolonged bath in aqueous (not alcoholic) iodine-iodide solution, followed by a less headlong rush through absolute alcohol.

A useful stain, which introduces a new principle into technique, is the celestine blue of Becker,¹⁰ reinvestigated by Proescher and Arkush⁴¹ and by Lendrum.⁴¹ Celestine blue B is an oxazine dye, related to brilliant cresyl and nile blues. By itself, in aqueous solution, it stains little except the ground-substance of cartilage, but when dissolved with iron alum it forms a remarkable soluble lake. One might compare this with the familiar solutions of haematein with alum, but there is a striking difference. Haematein is an acid dye whose combination with the basic mordant is not surprising. Celestine blue B is a basic dye (a chloride), and the formation of a lake with a basic mordant is not self-explanatory. One may perhaps look to the two OH groups in the molecule for the acidic tendency exhibited. The staining solution is violet, but it stains chromatin a pure blue. Its great advantages are that after appropriate fixatives it works rapidly but progressively, for a long time leaving the cytoplasm untouched, and that it shows complete resistance to extraction by alcohol. So far it has only been used as a routine histological dye for nuclei, but it would probably be useful as a chromosome stain. It does not work well after Bouin fixation.

Cytologists do not seem to have shown much originality in adding reducers of surface tension to their staining solutions. Aniline and phenol have often been used as 'accentuators', and it seems likely that reduction in surface tension is the explanation of their action. One might suppose that hexylresorcinol, for instance, might be much more effective.

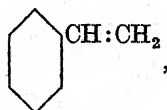
Many valuable dyes are very rapidly removed by alcohol, and it seems worth while to bring to the attention of cytologists an old but apparently almost forgotten method of circumventing this difficulty, much used by Unna and by Pappenheim.⁵⁷ The stained slide is dried by blotting and taken straight into a mixture of alcohol (or alcohol and acetone in equal parts) with 3 times its volume of xylene. During 5 or 10 minutes the alcohol completes the dehydration, while the xylene greatly reduces the speed at which the dye is extracted.

d. Mounting Media.

New synthetic mounting media are now available, some of which may possibly be found superior even to Canada balsam, whose virtues had seemed to place it almost beyond competition except for special purposes. Certain cycloparaffins are now available which have the necessary properties of ready adhesion to glass on evaporation of the solvent, transparency, lack of colour, neutrality, inertness, permanence, and refractive index (though it would not appear that their optical dispersion has been much studied). Groat²⁹ has found that for the cycloparaffin 'nevilleite V', also known as 'clarite', the most suitable solvent is toluene. A solution at 60 per cent. gives a convenient consistency, and

the material is used exactly as though it were a solution of balsam in xylene. The refractive index at the D line only differs by 0.009 from that of balsam, and the neutrality and absence of colouring with age are definite advantages. It remains to be seen whether experience will expose any defect in this promising medium, whose rather definite chemical composition commends it, though the fact that there is a variation of some 10° C. in the melting-point indicates that absolute purity has not been attained.

The styrene derivative 'distrene 80' has been recommended by Kirkpatrick and Lendrum,³⁵ who have studied the suitability as mounting media of a number of natural resins and synthetic substitutes. Styrene itself,



is a liquid which may be obtained from the plant product storax or synthesized in the laboratory. It polymerizes on heating to a glassy substance. The exact chemical nature of distrene 80 does not appear to have been disclosed by the manufacturers (Messrs. Honeywill and Stein, Ltd.), but it is stated to have the enormous molecular weight of about 80,000. It is readily soluble in xylene and the solution is colourless. A simple solution is not desirable, however, on account of the tendency for the medium gradually to shrink back under the cover-slip. Just as cellulose nitrate may be 'plasticized' or caused to retain its shape in mouldings by the addition of camphor, so another plasticizer, tri-cresylphosphate (a colourless liquid, or solid if perfectly pure), serves to prevent, or at least reduce, the tendency of distrene 80 to retract under the cover-slip of microscopical preparations. Kirkpatrick and Lendrum have shown that their plasticized distrene does not tend to change in pH on keeping, and the writer confirms their finding that even the blood dyes, which are very sensitive to changes in pH, retain their colours when this medium is used. He finds the further advantage, not mentioned by the authors, that the metachromatic colour given to mucin by certain thiazine dyes, which often gradually disappears in balsam, is well retained.

The writer would urge that the very general tendency to use mounting media approximating to glass in refractive index is overdone, and that much might be discovered if fluids of quite low index, including water itself, were more used. In particular he has been struck by the easy visibility of the lacunae in the cytoplasm of the egg of the cavy in sections while still in water, and their disappearance on mounting in balsam. Aqueous 'fluid mounts' are often used for such objects as whole Polyzoa, and there would not appear to be any reason why these

media should not also be applied to sections. The cover-glass could be cemented round its edges with the same substances as are used with fluid whole-mounts. Some preliminary studies have suggested that these methods will be quite generally useful for showing the vacuolar component of the Golgi complex, which is more difficult to see when the preparation has been rendered very transparent.⁷

e. Demonstration of Mitochondria and the Golgi Complex.

The most difficult cytological methods are probably those for mitochondria. Except where these cell inclusions are very large, as, for instance, in the convoluted tubules of the mammalian kidney, it is not easy to differentiate accurately the dye used to stain them. By far the most popular dye is acid fuchsin, which was introduced for this purpose by Altmann in 1886.³ Altmann later found it best to use the dye hot, and in this he is nearly always followed. His heroic differentiation in hot picric acid solution, however, of which he says mildly, 'Dieses letztere Erwärmen ist der schwierigste Theil des Färbungsverfahrens',⁴ is beyond the powers of most present-day cytologists. One prefers to follow Metzner⁵⁰ in using one's picric acid cold, or, better, to substitute another nitro-dye, aurantia, for the simpler picric acid and to use a cold alcoholic solution. This method of differentiating acid fuchsin, introduced by Kull³⁶ in 1914, is probably the best. Kull himself recommended staining the chromatin with thionine or toluidine blue, and this method often gives excellent results; but it is sometimes very difficult to get the blue stain sharply confined to the chromatin. A more recent modification is that of Volkonsky.⁷³ It is probably on account of its great complication that this method has been used by so few workers, though it is noteworthy that the great French cytologist Parat used it as his standard method for mitochondria in permanent preparations.⁵⁸ Volkonsky differentiates the acid fuchsin by Kull's method and then seeks to fix the dye in the mitochondria by the use of phosphomolybdic acid. The chemistry of the process would not appear to be understood. Volkonsky now proceeds to overstain everything heavily with a polychrome methylene-azure-violet solution, which he differentiates in a remarkable fluid of Unna's, namely, a solution of orange G in saturated aqueous tannic acid. This differentiation proceeds reasonably slowly and is easier to control than the rapid extraction of Kull's toluidine blue by alcohol. Beautiful results are given by this technique, which the writer strongly recommends. The mitochondria are, of course, red, and the cytoplasm yellow with orange G. (The aurantia, so useful as a differentiator, hardly survives the subsequent treatment.) Chromatin is blue-violet, and there is metachromatic staining of mucin.

Volkonsky himself gave a very short account of his method, and it will be found necessary to practise a good deal before perfection is

attained in carrying it out. The writer believes that in this, and in all other methods for mitochondria, it is best to choose somewhat arbitrarily certain definite times for which the slide is left in each stain, differentiator, stain-fixer, and dehydrating agent. The slide is taken right through to balsam and then critically examined under the oil-immersion lens. The examination reveals in what way the staining is particularly imperfect (and there nearly always are imperfections when a first attempt is made on a tissue with which one is not familiar). As a result of the examination, a decision is reached to lengthen or shorten *one* of the processes. For instance, if the acid fuchsin has tinged the cytoplasm, the aurantia bath will be made longer. A second slide is now run through to balsam, all the times with the one exception being exactly as before. The slide is critically examined, and imperfections again noted. It may be that the blue of the chromatin is scarcely deep enough. The decision is therefore reached to run another slide through with all the times the same as in the previous trial, but with a shorter time in the annin-orange solution (or, alternatively, in the absolute alcohol used in dehydration, which also differentiates the chromatin dyes). The resulting slide is examined with the immersion lens, and the whole process is repeated, if necessary, many times, until perfection is reached. It is obviously essential to have many slides, all carrying sections of exactly the same thickness; and the thinner they are, the easier it will be to make good preparations of the mitochondria. It will be found that time is saved in the long run if only one change is made at each trial, and each unsatisfactory slide must be thrown away and not brought back for restaining. Once one has discovered the best times for each bath with any particular tissue fixed in any particular way, one can make perfect preparations every time by simply adhering to the correct periods, which will of course be recorded for future use.

The Golgi complex usually presents less technical difficulty than the mitochondria, though those who are unfamiliar with the Golgi techniques often imagine them to be especially troublesome. Unfortunately there is no technique that will show the Golgi complex with certainty and evenly, through thick pieces of tissue. It would be worth while to try to evolve such a technique, which would be valuable even if it somewhat distorted the structure of the complex, just as the routine histological methods are valuable although they usually give a net-like structure to nuclei.

It may be remarked that no one knows what structure the ideal technique should show. The Golgi network is often spoken of, but it may be queried whether this is often a good description. If such a thing could be constructed as a true model of the complex, magnified several thousand times, and if this model were shown to a member of the

general public, it is possible that he would compare it to accumulations (or rows) of spheres embedded in a slimy looking material, or to spheres with bananas adjacent to them or touching their surfaces, or to spheres with separate strings intertwined among them, or even in some cases—if one may dare to suggest a heresy—simply to spheres; but the writer is doubtful whether the word network would often be used to describe the model, though it might occasionally be applicable.

Some writers have felt strongly that this or that component of the complex 'is' the Golgi element. Hirsch³² has answered such claims with dry wit by solemnly defining the *apparato reticolare interno* as, 'Mit Silber imprägnierbare, scheinbar netzartige Strukturen in den Purkinjezellen, in Zellen der Med. obl. und des Rückenmarks von Strix'. There have been those who have sought to define the true Golgi bodies as those which reduce silver nitrate or osmium tetroxide under certain conditions, but almost everything in the cell can be blackened by impregnations, and the decision when to stop impregnating or how long to reduce is a subjective one, dependent upon personal views as to what the Golgi bodies ought to look like in the final preparation. Once a structural concept has been formed in the mind, one is extraordinarily apt to work towards that concept, regarding preparations as 'good' if they show the preconceived structure and 'bad' if they do not. The writer will not willingly allow that the next man shows greater proficiency in the preparation of standard Golgi nets than he does; but he doubts whether the knowledge of how these structures can be made to appear has necessarily any close connexion with knowledge of how to make the Golgi complex reveal its true structure.

So as to avoid the possibility of misunderstanding, it must be repeated that the discovery of an easy method which could be relied upon to give some sort of figure of the Golgi complex evenly over fairly thick pieces of tissue would be a great advance in technique; but what is particularly wanted is a method (easy or difficult) for discovering the real structure and composition of each part of the complex.

The fact that no one understands the chemistry of the current methods is a severe warning not to identify any component of the complex as Golgi body or not Golgi body by reliance on the criterion of whether it blackens by the current techniques. As Da Fano²⁰ has said of his valuable method, 'In certain conditions which I have not yet been able to establish with sufficient precision it stains, besides the internal apparatus, intracellular formations, which according to their morphology and arrangement are to be considered as mitochondria.'

Why the addition of certain salts to the fixative should help the subsequent impregnation of part of the Golgi complex with silver is obscure, but the fact is not in doubt. It was Cajal¹⁶ who first discovered this fact. He found the nitrate to be the best acid radicle, and uranium,

manganese and lead were all suitable metals, but uranium was the best. Da Fano²¹ found cobalt nitrate even better.

Recently Aoyama² has preferred yet another metal and another anion. His discovery of the virtue of cadmium chloride is important, and his fluid may be the best of the fixatives for use before silver impregnations. He points out that cadmium chloride is very diffusible and stable, and he claims for it a greater affinity for lipides than the other salts used for the purpose. Aoyama does not pursue the subject, and one does not know upon what facts this statement was based. However, it may be suggested that the effect of cadmium in Aoyama's technique might be associated with the ability of salts of that metal to render phospho- and galactolipines less easily dissolved. It will be recollected that these substances, when pure, are insoluble not only in water but also, unlike other lipides, in acetone; but Ciaccio¹⁷ has shown that when other lipides or cholesterol are present, they can form colloidal solutions even in water. To make them resist extraction from tissues by acetone (and thus to separate them histochemically from other lipides), Ciaccio renders them insoluble by treatment with cadmium nitrate. It seems possible that the explanation of the value of cadmium in methods for showing the Golgi bodies may be related to its ability to render phospho- and galactolipines less readily extracted from tissues. If so, a glimmer of understanding seems to appear behind the empiricism of Golgi techniques.

f. Histochemistry.

It is refreshing to turn away from casuistical arguments as to whether a given object—rendered visible by incomprehensible methods—is, or is not, to be regarded as a veritable Golgi body, and to approach the cell from the standpoint of the exact science of histochemistry. One gains much by looking at the cell from the standpoint of substance rather than predominatingly from that of structure. Instead of asking oneself, 'What are the mitochondria like in this cell?' one might often with advantage put the alternative (though of course not identical) question, 'How are the lipines distributed in this cell?' Structure must not be neglected, but cytologists must think also in terms of substance. The old students of minute anatomy used to do so. With them, research into substance went hand in hand with research into structure.

Eighty and more years ago works on histochemistry were being written. Strangely in a way and yet comprehensibly enough, it was the discovery of the ease with which different substances could be stained in different colours in microscopical preparations that led histologists to concentrate their attention on structure rather than on chemical composition. It is so easy to stain chromatin or collagen or what one will in a special colour and thus reveal its disposition that one tends to be led

astray from the severe discipline which demands a chemical explanation of every colour test. A mass of knowledge sprang up as to how to stain various objects in various colours, and although a lot was known about dye chemistry, yet the chemical reasons underlying the tests remained and remain obscure and wholly unreliable as means for revealing the chemical composition of the objects stained. A result of this state of affairs is that at the present day many cytologists have not even troubled to show by histochemical tests that ordinary cytoplasm contains proteins, nor know what tests are applicable.

It is scarcely an exaggeration to say that a new era in histological and cytological technique started in 1936 with the publication of Lison's *Histochimie animale*.⁴⁴ The firm insistence on real chemical tests, to supplant the pseudochemical ones so much in vogue, separates this book from the writings which preceded it.

Before turning to strict histochemistry in Lison's sense, one may mention that the pseudo-chemical methods are often very valuable if there is no false suggestion that they are true chemical tests. One might as well refuse to acknowledge the greeting of one's friend in the street until he has proved his identity, as question the composition of spheres staining red with thionine in the appropriate part of what, by its structure and occurrence, is obviously a mucous cell. The mistake would arise if one were to say that an unknown granule in an unfamiliar cell consisted of mucin, simply because it stained red with thionine. Similarly one often recognizes unsaturated fat, in appropriate cells, by the reaction with osmium tetroxide, and collagen by the effect of light green following phosphomolybdic acid; but these are not histochemical tests.

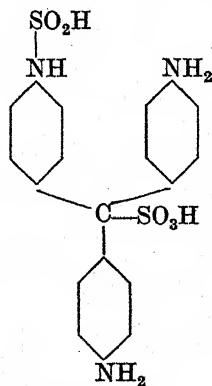
Instances of useful colour reactions of this kind could of course be multiplied. One that rather tends to be overlooked is Pappenheim's methyl-green-pyronine, which is very convenient for distinguishing between karyosomes and plasmosomes. Pappenheim at first⁵⁵ used rather vaguely defined proportions of the two dyes, but later he and others standardized certain proportions. The trouble with these mixtures is that they are not stable, and it is convenient to keep the pyronine solution separate from the methyl green until the day of use. One may dissolve pyronine G at $\frac{1}{4}$ per cent. in 20 per cent. aqueous glycerol and add $\frac{1}{2}$ per cent. of phenol. If one volume of 1 per cent. aqueous methyl green is added to 6 volumes of this solution, one has a mixture⁷ which stains karyosomes green or blue-green and plasmosomes red in half an hour or less. (Alcohol-xylene should be used for dehydrating: see p. 14.) This is a valuable method if no one claims that an unknown object is a nucleoprotein simply because it is stained green or blue-green by this dye. The chemistry of the process is not understood. Both dyes are basic, but if the proportions are as given above, the triphenyl-

methane dye stains chiefly chromatin and the xanthene dye other basiphil substances in the cell (and oxyphil ones to a lesser extent). Rhodamine S may be used instead of pyronine G (which is difficult to manufacture), but if this is done the dye should be made up at about 0.15 instead of 0.25 per cent.⁷

Enough has been said about these methods: they are good, but they are not histochemistry. If one wants to know whether an unknown object consists of or contains nucleic acid, one applies not Pappenheim's method but Feulgen's histochemical test.

Feulgen's test has been much used but less understood. It depends first on the fact that when thymonucleic acid is treated with warm hydrochloric acid the purine bases are separated off and thymic acid left behind. This substance gives the reactions of aldehydes, while thymonucleic acid before hydrolysis does not. At first it was thought that a sugar with aldehyde structure was responsible for the reaction, but it would appear⁴² that this is not so. A pentose sugar, without the aldehyde grouping, seems to be the constituent of thymic acid which gives the colour reaction with Schiff's aldehyde-reagent in Feulgen's test.

The reagent consists of the triphenylmethane dye, pararosaniline (or equally well rosaniline or the mixture of pararosaniline and rosaniline known as basic fuchsin), decolorized by excess of sulphurous acid. The colourless substance owes its lack of colour to the loss of the quinonoid linkage which existed in one of the three rings of pararosaniline. The chemical structure of Schiff's reagent is shown below. It should be compared with that of pararosaniline (p. 13).

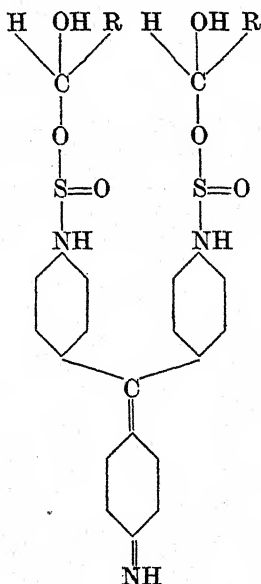


Schiff's reagent

If the excess of sulphurous acid is allowed to evaporate off (e.g., on one's fingers, as often happens), pararosaniline is re-formed and a brilliant magenta colour is produced. This, however, is not Schiff's reaction. To achieve the latter it is essential to have an excess of sulphurous acid throughout, until the reagent has been thoroughly washed out of the

tissue, in order to prevent the re-formation of pararosaniline, the appearance of which would give no histochemical information. It may be remarked that the most convenient way of obtaining sulphurous acid for Feulgen's test is by the action of hydrochloric acid on potassium metabisulphite.⁷²

When Schiff's reagent is added to an aldehyde or to the pentose sugar of nucleic acid, the following substance is produced (R being a radicle whose nature depends on the particular aldehyde or other substance which gives the reaction):



This has a magenta colour similar to that of pararosaniline, but unlike the latter substance it is formed without the necessity for sulphurous acid to be absent. It owes its colour to the re-formation of the quinonoid linkage.

If a substance in a cell gives the Schiff reaction although it has not been subjected to preliminary treatment with hydrochloric acid, then it is not thymonucleic acid. Lison⁴⁴ has especially stressed that it is essential to show that Schiff's reagent does not give the magenta colour unless there was a previous hydrolysis by hydrochloric acid. In the absence of this negative result, no sure conclusion can be drawn from a positive result with Feulgen's test, for aldehydes and other substances will give the reaction.⁴⁴ The cytoplasm of certain cells (e.g. in the adrenal gland and corpus luteum) also contains a substance called plasmalogen²⁵ which, after treatment with mercuric chloride or very prolonged acid hydrolysis, gives the Schiff reaction. This substance

is not thymonucleic acid. If these facts are kept in mind, one can tell, by the aid of Feulgen's invaluable technique, whether a substance occurring in a tissue of a living animal or plant does or does not contain thymonucleic acid.

The value of Schiff's reagent in histochemical technique is far from being exhausted when it has been used for demonstrating thymonucleic acid and plasmalogen. Baur⁸ has shown that it can be used in a histochemical test for complex polysaccharides, including glycogen. One cannot call Best's useful but empirical concoction a histochemical reagent for glycogen, because the chemistry—or perhaps one should say physics—of its reaction remains quite obscure; and the iodine methods are, in the writer's hands at any rate, troublesome. Baur's method is easy and precise. It depends on the fact that certain polysaccharides, including glycogen, can be oxidized by chromic acid to substances, insoluble in water and resistant to all ordinary processes of microscopical technique, which give the aldehyde reaction with Schiff's solution. One may fix in a fluid containing chromic acid, but the writer finds a very good fixative to be Rossman's alcohol-picric-formaldehyde.⁶⁵ The value of the alcohol contained in this fluid lies of course in the fact that glycogen is insoluble in it, but the other constituents require more explanation. It has been shown⁵⁹ that although picric acid does not precipitate glycogen, yet it fixes the cell proteins in such a way that the glycogen is firmly adsorbed on them; and the same probably applies to formaldehyde. If chromic acid was not used in the fixative, sections (cut in paraffin) are brought into an aqueous solution of chromic acid, and there the glycogen is rendered resistant to the subsequent treatment, which consists of the application of Schiff's reagent without any preliminary hydrolysis by warm hydrochloric acid. It is necessary to wash the reagent out with sulphurous acid solution, just as in the Feulgen technique, to prevent the re-formation of pararosaniline. If precautions are taken, a positive result definitely indicates the presence of a soluble polysaccharide. These precautions are the carrying through of two control slides, one of which is soaked in water instead of chromic acid solution, while the other is wetted with saliva before immersion in the chromic acid. If the body under investigation is colourless in these two control slides, but magenta after the standard Baur technique, it contains a soluble polysaccharide (e.g., glycogen).

Rather an obvious criterion of an ideal histochemical test, but one which it does not appear to have occurred to anyone to record in print, is that no process of differentiation should be involved. This is true of Feulgen's and Baur's methods, but not of every histochemical test. The Smith-Dietrich method for lipines^{70, 22} may be quoted as an example. The principle of this test is that lipines are oxidized by potassium dichromate, and a chromium compound is adsorbed on the oxidized

product. When sections are stained with haematoxylin without any further mordanting process, the lake is only formed in any considerable quantity where the chromium is held by the lipines. Oxidation by an alkaline solution of potassium ferricyanide differentiates the dye, removing it first from substances which have not adsorbed much of the metal, and subsequently from those which have. If one stops the differentiation at the right time, only the lipines are left black; everything else is grey or decolorized. This is a useful technique, and one is unlikely to make a mistake in differentiation; but it must be admitted that only a subjective idea (whether one's own or derived from the experience of others) can indicate when differentiation should be stopped.

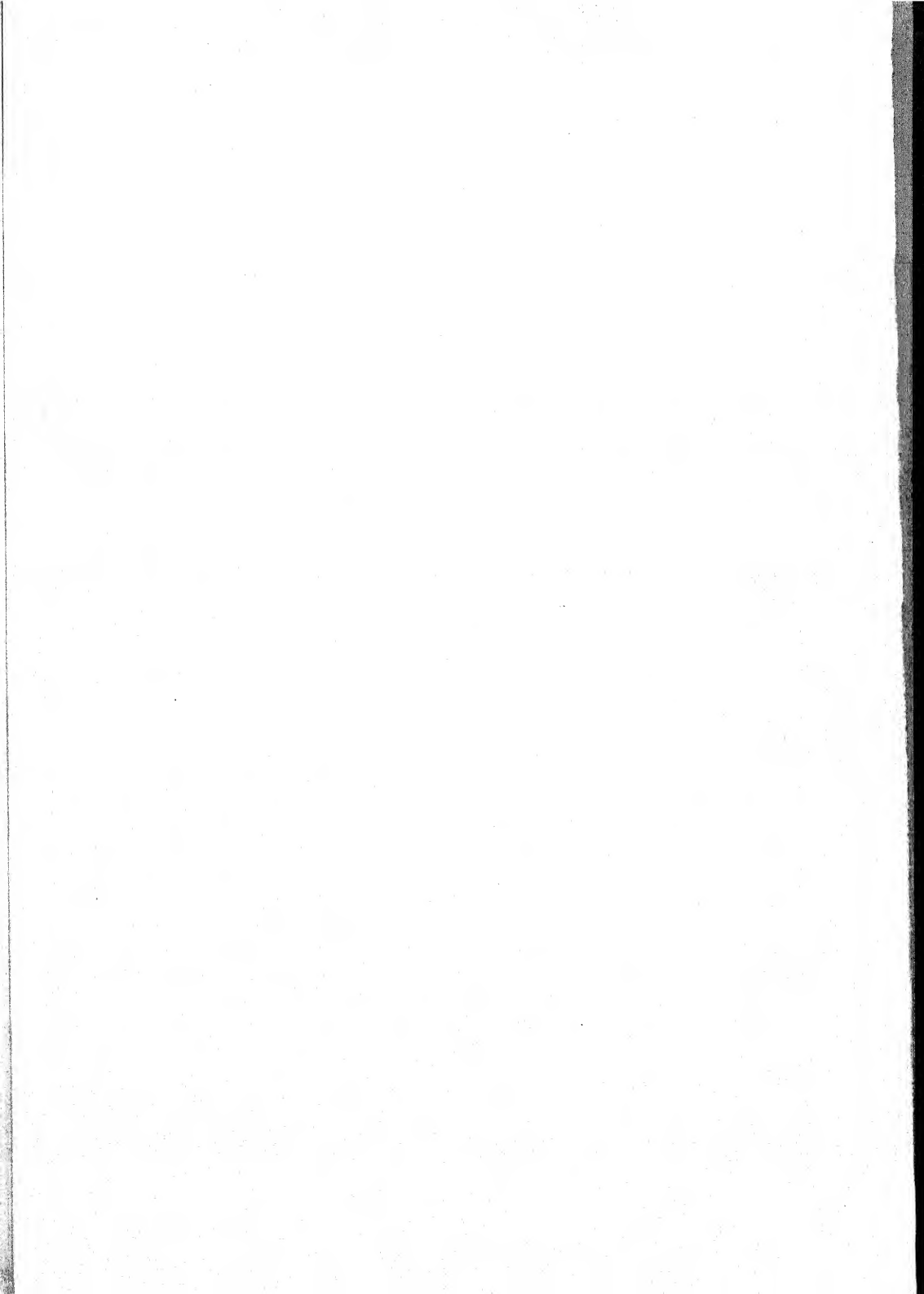
More complete histochemical study has probably been devoted to the spermatozoon⁴⁸ than to any other cell. It is desirable that an equally profound study should be made of less highly specialized cells. Now that we know, thanks to Lison⁴⁵ and others, that lifelike fixation is by no means incompatible with the application of histochemical tests, the student of biology might with advantage be taught to look at the cell from the histochemical standpoint as a matter of course, and to concentrate on thoroughly understanding a few processes which will give real insight into the substances which he examines under the microscope, rather than on acquiring a knowledge of how to apply a multitude of different recipes as empirical as the contents of an old-fashioned cookery-book.

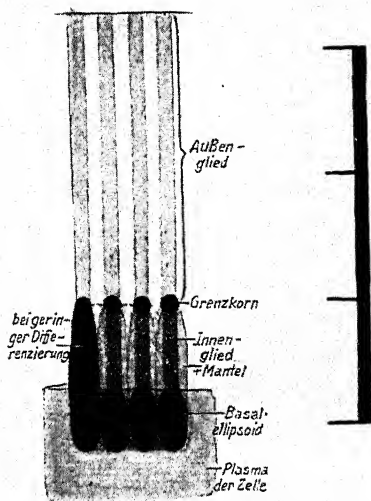
iv. MICROSCOPY

Whatever the nature of a cytological preparation, it can only fully reveal itself if it is examined by someone who understands the proper use of the microscope. Those cytologists who have not done so would profit from submitting themselves to the illuminating experience of studying the striations of Diatoms near the limit of optical resolution. Although most cytologists are naturally skilful microscopists, yet there are those who will not trouble to master the use of their instrument, but think that the only necessity is to buy the most expensive instrument possible, preferably with a foreign name, provided exclusively with apochromatic objectives. Optical resolution is more dependent on understanding than on expenditure. To buy a good microscope and use it with the condenser out of focus or inaccurately centred or with the aperture too far cut down is like buying a Rolls Royce and driving it uphill with the brakes on.

Lack of understanding results not only in less being seen than might be, but also in more being claimed as seen than could conceivably be. An example will make this statement concrete.

It will be recollected that the intestinal epithelial cells of vertebrates





A. Clara's diagram of the striated border of the intestinal epithelium of birds. A scale, 3μ long, is fitted beside Clara's diagram.



C. Clara's diagram, reduced to the magnification of the photograph of *Amphipleura pellucida*. Note that if one magnifies this figure by a hand-lens, further detail becomes visible. This could not be so if the diagram were genuine, for magnification beyond 1,600 diameters is empty and incapable of revealing further structure.



B. The Diatom *Amphipleura pellucida* $\times 1,600$. This magnificent photograph is taken from E. J. Spitta's *Microscopy: the Construction, Theory and Use of the Microscope* (1920), by kind permission of the publishers, Messrs. John Murray. By the use of oblique blue light and an apochromatic objective of the highest quality, Spitta has resolved the striations very clearly into dots. Resolution of such minute detail can only be obtained with very favourable objects, such as diatoms, and by the adoption of special methods. A scale of 10μ is fitted beside the photograph. Further magnification would be empty (i.e., could not reveal further detail).

The Plate illustrates how an author sometimes claims to see structures which are below the limit of microscopical resolution.

J. R. Baker

present a striated border to the lumen of the gut. The existence of the striations as tangible morphological entities is denied by Thanhoffer⁷¹, on the ground that he can pull the border about with micro-dissection needles without splitting it neatly down the striations. It is hard for anyone who has seen the striations—visible alike in fresh and prepared cells with critical conditions of observation—to accept Thanhoffer's denial. If one refuses to accept it, there is no need to rush to the other extreme of believing in the existence of the minute detail exhibited in Clara's diagram¹⁸ of the striated membrane in the epithelial cells of the gut of birds.

The accompanying figure (Plate 1) shows Clara's diagram at the original magnification, and beside it there is a scale which is based on the assumption that the striated border is $3\frac{1}{2} \mu$ thick, which is probably a generous allowance. In the figure it will be seen that the *greatest* thickness of the 'Mantel' (i.e. the greatest distance between the surface of the latter and the 'Innenglied') is less than 0.1μ , and it thins out delicately at each end! We are here in the realms of fancy. The details belong to that category of objects which have never yet been seen, but of which we may say, with Belloc¹¹, that 'Scientists, who ought to know, assure us that they must be so'.

A photograph of the Diatom *Amphipleura pellucida* has been reproduced on the same plate. This photograph shows part of the Diatom magnified 1,600 diameters, beyond which all magnification is empty and incapable of revealing further structure. Anyone who has succeeded in getting as good a view of *A. pellucida* as that shown in the plate will be aware that very good resolution has been attained. The dots are so close together that they are not very far from the limit of microscopical resolution. (It will be recollected that the diffraction haloes made evident by the ultra-microscope are not true images). Clara's diagram has been reduced to the same magnification as the *A. pellucida* and reproduced on the same plate.

For getting reliable information with the microscope, accurate optical alignment is a prime necessity. A seemingly troublesome but actually very convenient means to this end is the Sloan objective-changer. This would be an intolerable nuisance on one's staining or routine instrument, but on the microscope used for ultimate analysis of structure it is preferable, in the writer's opinion based on the experience of many years, to the revolving nose-piece. Each objective can be exactly centred to the optical axis of the microscope, and when in position is held as though by a vice. One soon learns to change the objectives quickly and without having to think about the process, and there is the great advantage that what was in the centre of the field of view before will be exactly in the centre when the change has been made, whichever objective may have been chosen. It is a similar convenience

to have each of one's condensers on a separate slide, with centring screws of its own (for instance, the apparatus of Messrs. W. Watson). The writer has found it convenient to put two crossed hairs in balsam on a microscopical slide, and to record on the label of the slide the vernier reading of the mechanical stage when the point of crossing is in the optical axis. Every few months the centring of all the objectives in their Sloan mounts and of the condensers in their Watson slides is checked by reference to the slide holding the crossed hairs, which is kept permanently. Any small departure from exact centring is easily corrected. By this means one obtains the consolation of knowing that whatever condenser one uses with whatever objective, no fiddling at all is required to attain optical alignment. The preference for this method is, of course, a personal one, and there are those who would rather be able to snap round the revolving nose-piece and would find the Sloan objective-changer rather cumbersome to manipulate. The writer believes, nevertheless, that there are many cytologists who would never go back to the revolving nose-piece on their best instruments if they had once got accustomed to the Sloan apparatus.

A useful adjunct to microscopical work which has recently become available to microscopists (through Messrs. W. Watson) is the transparent sheeting known as 'polaroid', by means of which polarized light is very conveniently obtained. The polarizing material consists of crystals of quinine sulphate periodide. They are extremely minute: indeed, there are said²³ to be 10^{12} of them in each square inch of the nitrocellulose film in which they are embedded, and they are beyond resolution by ordinary microscopical methods, so that the film appears homogeneous even when highly magnified. By an ingenious mechanical process the crystals are all orientated in the same direction. The nitrocellulose film containing the crystals is mounted between two sheets of glass, and the final thickness is only about 3 mm.

Polaroid is of a transparent pale grey. A circular piece fits into the standard screen-carrier below the condenser of the microscope, and another in a simple rotating holder above the eyepiece. A condenser with a large back lens presents no difficulty when polaroid is used, and the expenditure necessary to cover a big area with a Nicol's prism is avoided. Further, there is no need of any special mounting to hold the polaroid below the condenser nor any inconvenience due to lack of space between condenser and mirror. The eye can easily be brought conveniently near to the eyepiece, as the sheet of polaroid used as analyser is so thin. All these are great advantages, and polaroid is most helpful in determining whether doubly refracting substances are present, since the usual set-up of the microscope is retained and the turnover to polarized light is made in a few moments. The highest powers may be used, but if so a rather intense source of light is necessary. With crossed polaroids there is

nearly, but not quite, complete extinction of light from the background. Anyone who wishes to make careful measurements of angles of rotation, &c., will continue to use the more elaborate older methods for obtaining polarized light, but polaroid should find a place among the tools of the cytologist.

That most delicate tool of the cytologist, his cerebral cortex, is sometimes a source of error in the interpretation of minute structure. White⁷⁵ has pointed out that the direction of the spiral twisting of chromosomes may be misinterpreted for a curious reason. A left-handed spiral may be mistaken for a right-handed one simply because the out-of-focus parts of the chromosome are automatically referred by the observer to the more distant part of the field, behind the parts that are sharply focused, whereas actually they may be on the near side, above the focused field. One must be on one's guard against what might be called psychological errors of this kind. Not every cytologist remembers how extraordinary is the viewpoint obtained with lenses of high numerical aperture. One tends to forget that one's eye is in among the things one is examining, instead of nearly a foot away, and that an object lying beyond a small opaque obstruction may be rendered clearly visible by the widely divergent rays proceeding from it.

There has not been sufficient space in this chapter, nor experience in its author, for a critical discussion to be made of certain of the most interesting recent developments in cytological technique. No two cytologists would have chosen the same topics out of a wealth of material that is coextensive with cytology itself. The writer hopes that he may have succeeded in doing some small service to the movement towards thinking in terms of substance and of preferring histochemical methods to empirical ones. He hopes also that this movement will never lead anyone to think structure unimportant. He acknowledges his debt to Professor E. S. Goodrich, F.R.S., in whose Department he has worked for more than two decades and whose accuracy in the delineation of structure is so well known.

Note to p. 11, line 21:

Lison and Fautrez (1939, *Protoplasma*, 33, 116) account for metachromasy in another way.

CHAPTER II

PHYSICAL AND PHYSICOCHEMICAL STUDIES OF CELLS

PART I. GENERAL

By J. F. DANIELLI

i. INTRODUCTION

IN this chapter typical examples are given of the results of investigating cells by physical methods. Our endeavour is, not to be exhaustive, but to show the type of questions which may be answered by physical methods and to indicate some of the limitations of these methods. In conclusion we shall discuss the use of models of biological systems, again not exhaustively, but mainly in the light of relatively new knowledge of colloids and surfaces. In doing so we do not wish to convey the view that similarity of behaviour of biological systems and certain models affords any large measure of plausibility in concluding that the underlying mechanisms are the same in the two cases, but rather to show how the feasibility of hypotheses about biological behaviour may be tested, in the absence of sufficient detailed knowledge of the biological system for proof of existence to be obtained. For example, when we postulate that the plasma membrane of an *Arbacia* egg is a spherical shell of lipoid material, we are assuming that such shells are stable. This assumption must be tested by the construction of artificial spherical shells of lipoid. That artificial shells are stable shows us that the postulated structure is a *possible* one, though it tells us little about the probability of this structure actually existing in the egg.

ii. RADIATIONS and LIGHT

a. The Use of Radiations, &c.

The earliest observations of cytology were made with visible light, and simple visual microscopy is too familiar to need discussion here. More recently use has been made of polarized light, ultraviolet light, X-rays, and electron beams. The necessity of using these different forms of illumination is thrust upon us by the fact that a particle cannot be discerned if its diameter is less than about half the wave-length of the form of radiation employed. Thus the theoretical limits of particle size which can be discerned are roughly: visible light $0.25\ \mu$, ultraviolet light $0.1\ \mu$, electron beams $0.01\ m\mu$, X-rays $< 0.01\ m\mu$.* Owing to

* These magnitudes may be compared with the diameter of, e.g., red cells $10\ \mu$, *B. coli* $2\ \mu$, vaccinia virus $150\ m\mu$, tobacco mosaic virus $30\ m\mu$, ovalbumin molecule $5\ m\mu$, cholesterol molecule $1.0\ m\mu$, water molecule $0.3\ m\mu$.

practical limitations electron beams can at present be used down to $4\text{ m}\mu$ only, but as X-rays are useful up to the limit of $50\text{ m}\mu$, the whole range from macroscopic particles down to molecular dimensions is now open to study. In addition, substances transparent to one wave-length may be opaque to another, so that the use of, for example, ultraviolet light may show that a region appearing homogeneous in visible light is in fact not homogeneous.

The use of ultraviolet light extends the range of vision by comparatively little, but nevertheless has shown some protoplasmic structures invisible in ordinary light; an important example is in the microphotographic study of chromosomes. Electron microscopy is in its infancy, but a practical instrument has now been evolved and it is only a matter of time before very valuable results will be obtained. Unfortunately there are great difficulties besetting the use of electron beams for living organisms, owing to their high water content. Water absorbs electrons very strongly.

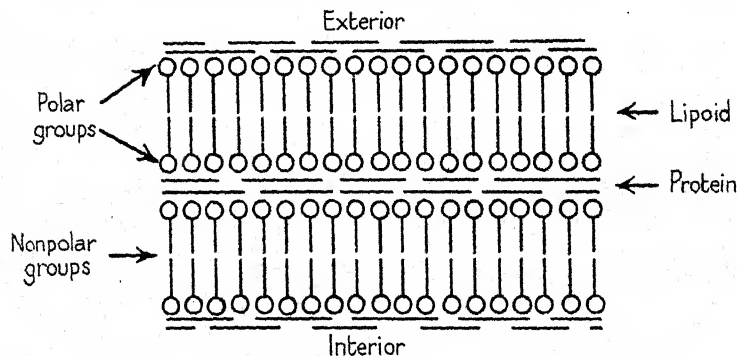
The purpose of using polarized light is rather different: it enables us, though still using visible light, to detect birefringent regions,* i.e. regions in which there are certain types of regular molecular or micellar patterns. Picken has recently reviewed this field.† Concentrated suspensions of some viruses are spontaneously birefringent. Birefringence studies allied with X-ray studies show that in such suspensions the virus particles are needle-shaped and the needles are oriented parallel to one another (Bernal and Fankuchen)¹⁰. It has been shown that chromosomes of many species are birefringent, and Schmidt¹²⁵ showed that the chromatin micelles of the chromosomes of *Psammechinus miliaris* are negatively birefringent, but that the micelles are arranged in the chromosomes in such a way as to make the chromosome positively birefringent with respect to its long axis. The mitotic spindle and asters are birefringent, and the birefringence of the spindle is not lost on damaging the cell until the spindle swells. This suggests very strongly that the spindle is an aggregate of long micelles orientated parallel to its length—a view which is supported by the fact that on dehydration the spindle changes in breadth, but not in length.

The muscle fibre contains myosin, a protein which is threadlike and is known to show birefringence *in vitro* when in a flowing stream, due to orientation of the needles parallel to the line of flow. This protein is generally believed to be responsible for the birefringence of the muscle fibre, being oriented parallel to the fibre axis. The striation of the fibres of striped muscle is believed to be due to alternating regions of orientation, the myosin in the isotropic regions being oriented at random and

* Regions in which molecular orientation or particle orientation occurs are, under appropriate conditions, optically anisotropic (see Lowry¹⁰¹).

† Applications to the cell surface are included in Chapter III.

in the anisotropic regions oriented parallel to the fibre axis (Muralt¹¹⁶). Bernal⁹ has suggested that in the apparently isotropic region (non-birefringent) the molecular packing is closer than in the anisotropic regions, and the direction of orientation is at an angle to the fibre axis. The birefringent (i.e. anisotropic) regions diminish in proportion and the isotropic regions increase in proportion, during contraction (or, according to Bernal, the proportion of molecules orientated at an angle to



TEXT-FIG. 1. Structure of myelin sheath of a nerve fibre.

the fibre axis increases). X-ray studies of contraction have not yet proved very fruitful, though some changes have been observed which possibly occur in the myosin molecule during contraction (Astbury²).

Birefringence has also been observed in other contractile tissues, including cilia and pseudopodia. It is probably true that most protoplasmic movement is associated with changes in the arrangement of needle-shaped colloidal molecules.

Schmidt and Bear¹²⁷ have shown from the optical properties of the myelin sheath of medullated nerve that the myelin consists of layers of lipid molecules, oriented radially with respect to the nerve axon, and layers of protein molecules oriented tangentially. It is probable that the lipid molecules are arranged in concentric cylindrical shells, two molecules thick, separated by layers of protein (Text-fig. 1).

A similar lipid-protein structure is believed to exist in the rods and cones (Schmidt¹²⁶), in this case with the sheets of molecules at right-angles to the long axis of the cells. This lipo-protein structure consists of a repetition of the basic structure—a bimolecular lipid layer stabilized by adsorbed protein—suggested by Danielli³⁹ for the cell membrane. These types of structures found in myelin and in the rods and cones may thus arise directly from the cell membrane.

b. Mitogenetic Radiation.

Gurwitsch (1923; see review 1932)⁵⁶ claimed that some tissues emit a specific radiation which induces mitosis in other tissues. It was

claimed that these radiations were in the ultraviolet, around $220\text{ m}\mu$ in wave-length, and the school of Gurwitsch has since claimed that mitogenetic radiation is closely associated with many other forms of cellular activity. The great majority of workers in this field have been unable to substantiate Gurwitsch's claims and most workers now discredit the existence of this specific radiation.

c. *The Spectroscope.*

Use of the spectroscope is limited, but where a process involves a change in the adsorption spectrum of a cell it is an extremely useful instrument. Important instances of its use are the work of Keilin⁸⁷, who was able to observe the intracellular oxidation and reduction of the cytochrome enzyme system, the work of Millikan on the intracellular oxidation and reduction of muscle haemoglobin and the work of Irwin⁸² on the absorption of dyes by plant cells. For the use of the spectroscope in the analysis of the ash of micro-incinerated tissues see Chapter VI.

iii. MECHANICAL INVESTIGATIONS

a. *Centrifugal Force.*

The Cell Membrane. The use of centrifugal force in the study of the cell membrane is discussed in Chapter III.

The Viscosity of Protoplasm. The viscosity of a fluid may be determined by studying displacement of a particle due to Brownian motion, or from the rate of movement of a particle in a centrifugal, gravitational, electrical, or magnetic field. Of these possible methods, centrifugal force and Brownian motion have proved the most satisfactory. Table I shows values for the viscosity of the cytoplasm of a number of different cells. Usually the values obtained are not greater than ten

TABLE I. *The viscosity of cytoplasm (in centipoises)*

Viscosity of water = 1 centipoise
 " " olive oil = 100 centipoises.

Species	From Brownian movement	From centrifugation	Author
<i>Spirogrya</i> . .	2-47	..	Baas-Becking, Bakhuyzen and Hotelling, 1928 ³
<i>Arbacia</i> egg . .	4	2	Heilbrunn, 1928 ⁷⁰ , 1926 ⁶⁹
<i>Amoeba</i> . . .	6	2	Peckarek, 1933 ¹²⁴
			Heilbrunn, 1929 ⁷¹
<i>Sabellaria</i> egg .	20	..	Harris, 1935 ⁶⁷

times that of water, though in exceptional cases values as high as 8,000 have been obtained (Fetter⁴⁶, for *Paramecium*). Where an apparently very high viscosity is obtained, it is probable that intracellular stream-

ing, or intracellular structures, are interfering with the free movement of the particle under observation. The low value obtained for the cytoplasmic viscosity of most cells strongly suggests that the cytoplasm does not have a rigid invisible structure. Gray⁵⁴ found that the nucleoli of the large nucleus of *Asterias* or *Echinus* oocytes move freely through the nucleus under gravitational force, always coming to rest on the 'floor' of the nucleus. From the rate of fall it was calculated that the viscosity of the nucleus was 10 times that of water.* It follows, therefore, that in this case also there can be little in the way of a rigid, permanent structure extending throughout the nucleus. Harris (1939) also studied the fall of the nucleolus of the *Echinus* oocyte under gravity. He concluded that the nucleus is a homogeneous thixotropic fluid, of viscosity, calculated from Brownian movement, *ca.* 10 times that of water. In addition, it was found that there is a force acting on the nucleolus, possibly electrical, which is constant in magnitude and direction for a given oocyte. This force produces secondary movement of the nucleolus in a direction independent of the orientation of the oocyte. The force is abolished by M/500 KCN.

On the other hand, using the centrifuge microscope, Harvey and Marsland⁶⁶ have shown that, whereas most of the particles observed move at a uniform rate in a centrifugal field, others show intermittent jerky motion, and it was concluded that the cell is in a state of incipient gelation, and that rapidly reversible sol \rightleftharpoons gel changes are occurring in local regions of the cytoplasm. There is usually a semi-permanently gelled region close to the plasma membrane (the cortex, or ectoplasm).

Studies of the change in cytoplasmic viscosity during cell division have been made, particularly by Heilbrunn in 1921⁶⁸. There is a rise in viscosity associated with the development of the sperm aster, followed by a fall, then by a second rise associated with development of the amphiaster. These results have been confirmed, qualitatively, by estimates of viscosity made by Chambers¹⁸ using the micro-dissection technique. In death most cells set into a gel and, as Harvey⁶⁵ has remarked, the action of stimuli of various kinds is to cause an increase in viscosity, culminating in irreversible gelation if the stimulus is sufficiently strong.

Centrifugal force has remarkably little action on the development of cells. A striking example of this is the work of Beams and King^{8A}: *Ascaris*^{8A} eggs were centrifuged for 4 days at 150,000 times gravity, and still developed normally when removed from the centrifuge. A great deal of work has been done on the effect on development of centrifuging the visible structures of the cell into abnormal positions, e.g. by Boveri (1901)¹⁴, Lyon (1907)¹⁰³, F. R. Lillie (1909)⁹⁵, Morgan (1910)¹¹⁵, Conklin (1917)³¹. The general conclusion is that displacement of the visible

* Heilbrunn⁷⁰, corrected by Harris (1939). Several assumptions, including the difference between the densities of nucleus and nucleolus, are made.

bodies does not affect the polarity of the egg, the cleavage pattern, or organ formation. It is not yet clear whether very high centrifugal forces, capable of causing sedimentation of the dissolved proteins of the cytoplasm, will have contrary results; though this is rather to be expected. Costello³² has found that if *Arbacia* eggs are centrifuged before fertilization, until they finally divide into two halves (one light, the other heavy), practically no fertilization membrane appears on the light half and a very marked membrane is found on the heavy half. Evidently centrifugal force can shift a substance or substances connected with the elevation of this membrane.

The technique of centrifuging eggs into halves (and then into quarters) has been developed very largely by E. N. Harvey⁶⁴. When centrifuged, stratification occurs, oil droplets moving to the centripetal end of the cell, and heavy granules, &c., to the centrifugal end. The tendency of these strata to move in opposite directions elongates the cell, which spontaneously divides into two when its length is π times its diameter. This is a particularly useful technique. Recently studies have been made of the respiration of light and heavy halves (Shapiro¹²⁸) and of the distribution of enzymes between the light and heavy halves (Holter⁸⁰).

One of the most important discoveries made by this technique is the observation of parthenogenetic merogeny in *Arbacia* egg halves by E. B. Harvey⁶³. Halves without nuclei, when subjected to parthenogenetic reagents, cleave in a relatively normal manner and will continue to do so up to the stage of a blastula of some 500 cells. That nuclei may divide and meiosis occur without cell division has been known for many years. Mrs. Harvey's observation shows that cell division may occur independently of nuclei and that therefore the minimum requirements for cleavage are contained by enucleated cytoplasm.

b. Micro-dissection and Micro-injection.

Micro-dissection and micro-injection operations, carried out on single cells, have provided a wealth of valuable information. The two chief objections or difficulties (apart from the development of the necessary manual skill) are that (1) operations on cells instantly make them abnormal; (2) conclusions reached are necessarily relatively subjective, especially when questions of detail are involved. The first difficulty is the more serious: when an organ is removed from an animal, say a dog, we have very many ways of estimating the degree of abnormality introduced. When a single cell is operated upon we have as yet only rather crude criteria of abnormality; if cytolysis occurs, or gelation, or permeability to dyes is abnormal, if pigment granules and other cytoplasmic inclusions look abnormal, or if in the development of an egg abnormalities develop, we know serious damage has been done. Beyond this it is hardly possible to go. Thus, if mammalian physiology were

exposed to the same restrictions as cell physiology we should have to judge the results of an operation by whether the dog died, was apathetic or normal in movement, whether it passed into rigor or tetanus, whether the disposition of vital stains was abnormal, whether its hair fell out, and whether its puppies showed gross anatomical abnormalities.

The major contributions to technique have been made by Chambers and by Peterfi. A fairly detailed description of results obtained up to 1924 is given by Chambers¹⁹.

Many cells are surrounded by a tough elastic pellicle (the vitelline membrane); this may be dissected away from the cell. For example a slit may be cut in the pellicle of an *Arbacia* egg, through which the egg may be expressed without apparent damage to the plasma membrane. The surface of such a naked cell is readily wetted by oils (Chambers and Kopac²⁹) and appears to be liquid. It can be torn by a sharp movement of a dissection needle, in which case the cytoplasm usually pours out and mixes with the surrounding fluid; substances normally unable to enter a cell do so readily through such a tear. But a needle moved carefully passes into the interior of the cell without breaking the membrane. The membrane of most cells is relatively tough and has marked elasticity: an erythrocyte may be stretched between two dissection needles to a length several times its normal breadth and will recover its original shape on release.

The interior of the cytoplasm is usually fluid and of low viscosity, except for a cortical layer (the ectoplasm) which is jelly-like in consistency and usually contains few inclusions. There is some evidence that this layer is intimately concerned in cell cleavage; a fragment cut from an *Asterias* egg will develop on fertilization in a relatively normal manner if a sufficient fraction of the cortex is included in the fragment. If there is little or no cortical material the fragment will not cleave, even if it contains the egg nucleus. *Amoeba* pseudopodia, when pinched off from the main body of the amoeba by a needle, retain ability to move and to ingest particles for a considerable time. If a small volume of fluid (water or salt solution) is injected into an amoeba it usually appears to mix readily with the cytoplasm and the excess water is eventually expelled through the cell membrane. Otherwise local liquefaction of the cytoplasm may occur and the liquefied area frequently rounds up and then resembles a vacuole. If a large amount of water is injected the amoeba may be killed; if it eventually recovers, a large part of the injected fluid may be pinched off in a blister. Basic dyes injected cause local coagulation of the cytoplasm, the coagulum eventually becoming dispersed through the more fluid cytoplasm as coloured granules. Acid dyes frequently cause liquefaction of the cytoplasm and the liquefied area may be pinched off.

The mitochondria of marine eggs appear, when in the rod-shaped or

filamentous form, to be relatively solid, sticky, elastic bodies. The pH of the fluid cytoplasm of most cells is about 7.0, as given by injected acidic indicators (which are likely to be more reliable than basic indicators) and the nucleus, where its pH can be measured, is usually 0.5–0.6 pH units more alkaline.* Similar results have been obtained with vital stains. Studies on the oxidation-reduction potential were first made by Needham and Needham¹¹⁷, and have been extended by Chambers and his colleagues.

The nucleus shows quite different properties according to whether it is in the resting or active condition. The resting nucleus appears in most cases to be liquid. Tearing the nuclear membrane causes disintegration of the nucleoli, followed by liquefaction of the cytoplasm and possibly by cytolysis. The liquefaction of the cytoplasm spreads from the nucleus outwards. When transposed from the interior to the exterior of a cell through a tear in the plasma membrane the nucleus may swell to bursting, or may set to a rubbery gel.

According to Chambers, the active nucleus varies in its behaviour according to the stage of development attained. When the very early prophase nucleus (of grasshopper spermatocytes) is injured, filaments appear in the nucleus composed of granules adhering to an elastic hyaline thread. These shorten and thicken, with apparent fusion of the granules, into rods reminiscent of chromosomes. One of the nucleoli also gives rise to a similar body. If injured at a slightly later stage, typical prophase chromosomes appear rapidly, and if prophase chromosomes are already visible inside the nucleus, pricking the membrane causes an increase in definition and transition to metaphase chromosomes. Individual chromosomes may be pulled away from the spindle during mitosis. Such detached chromosomes are elastic bodies with a cortex apparently different from the (hyaline) central core.

The spindle varies in viscosity and gel-like properties, both with time and with species. It may be a stiff gel, or relatively fluid. The amphister may have sufficient rigidity to distort the shape of the cell (Gray). The sperm aster of starfish and sea-urchin eggs is gelled, but the centrosphere (in the centre of the aster) is fluid; the gel is most rigid close to the centrosphere and becomes weaker the farther away from the centrosphere (Chambers). Stirring the interior of the cell may cause the amphister to disappear, but it will reappear on cessation of stirring if the agitation is not excessive. In nuclear division the two asters are firmly united by the spindle until elongation and division of the spindle occurs, after which the asters are readily moved independently.

Thus micro-dissection shows up two main features of cell structure: (1) the role of the membranes, (2) the texture of the various con-

* It is possible that this difference is fictitious and is entirely due to the protein error being different in the two cases, for the cytoplasm and nucleus contain quite different proteins.

stituents. A certain amount of light is also thrown on many other features of cell physiology.

It will have been observed that the parts of the protoplasm concerned in movement are capable of gelation and are elastic. We have previously noticed that the same structures are also optically anisotropic. We shall later discuss the significance of these properties.

Before passing to a fresh topic, the work of Landis on blood-capillary permeability must be mentioned. Landis^{89, 90, 91} was able to measure capillary permeability in absolute units by adapting the micro-dissection technique, and so has obtained a great deal of useful information about the behaviour of the cells forming the capillary walls. A capillary was chosen and closed by pressure from a needle applied toward the venous end. Then the movement of a red cell in the capillary, anterior to the block, was observed with an ocular micrometer. Movement of the red cell occurs as fluid is filtered out through the capillary wall, under the hydrostatic pressure in the capillary, and is proportional to the volume of fluid filtered out. The dimensions of the capillary are measured and the hydrostatic pressure inside the capillary obtained by cannulation with a micro-cannula. From these results the volume of fluid filtered off per μ^2 per second per atmosphere pressure can be obtained.

An important recent development is the use of the Cartesian Diver for micro-metabolism experiments.* By this means the study of the metabolism of single cells, or of groups of small cells, removed from, for example, an invaginating blastula by micro-dissection, is much facilitated. Needham and his colleagues have made an extensive study of morphogenesis and metabolism, using this technique.¹¹⁸

c. Pressure.

Reducing the pressure on an organism below atmospheric pressure has little effect until asphyxiation commences. Increasing the pressure above a limit, critical for a given organism at a given time, has the effect of liquefying the plasmagel and so introduces most profound changes in the behaviour of cells. The physico-chemical basis of this liquefaction is not clear and we shall therefore not discuss it, but will simply remark that the elucidation of the mechanism of such liquefactions is of outstanding importance.† Many other physiological changes are observed.

The general result of liquefaction is the interruption or suppression of all forms of behaviour involving active protoplasmic movement, and sufficiently high pressures may cause death, possibly due to denatura-

* Linderström-Lang⁹⁹; Linderström-Lang and Glick¹⁰⁰.

† It is possible that liquefaction⁷¹ is due to increased hydration of protein molecules; but as Cattell¹⁷ remarks, 'It would be difficult to eliminate completely the possibility that the changes in viscosity are not secondary to changes resulting from stimulation or chemical reactions.'

tion of proteins, possibly due to the disintegration of essential organized systems. The lower pressures sometimes cause enhancement of activity.

Grundfest and Cattell (1935) found that, with single nerve-fibres, pressures between 300 and 500 atmospheres increase the action potential by 10 per cent. and the duration by 20 per cent. As the pressure is raised towards 1,000 atmospheres the potential falls off, the rate of conduction is slowed, and finally the response disappears. All the changes are reversible.

With muscle (see review, Cattell, 1936¹⁷) the sudden application of 300–400 atmospheres to a resting muscle causes a small contracture. There are three apparently distinct effects on the behaviour of electrically stimulated muscle: (1) relatively low pressures cause the liberation of more energy, shown by an increase in the initial heat production and an increment in the tension of twitch; (2) at higher pressures the response is both slower and weakened, suggesting a decrease in elasticity and possibly an increase in viscosity also; (3) finally, at a sufficiently high pressure (c. 1,000 atmospheres), contraction is abolished. If in phase (2) the pressure is suddenly released during the development of a twitch, the twitch is augmented above the value which would have been obtained if the whole operation had been carried out at atmospheric pressure; this suggests that the increase in mobilization of energy found at the lower pressures persists at least during the second phase. The mobilization of this energy is complete in the first eighth of the contraction process (Brown¹⁶).

Marsland and Brown¹⁰⁸ found that 400–600 atmospheres was sufficient to cause liquefaction of the plasmagel of *Amoeba proteus* and of *Amoeba dubia*, with consequent retraction of pseudopodia and rounding up into a roughly spherical shape. Kitching and Pease⁸⁸, however, found that the very long tentacles of the suctorian protozoan *Ephelota coronata* may resist 800 atmospheres without liquefaction. As mentioned previously, structures with liquid surfaces and no rigidity spontaneously divide when their length exceeds π times their diameter; thus the tentacles of *Ephelota* would break into a number of small droplets if liquid and are only prevented from doing so by the rigidity of the plasmagel. When the plasmagel liquefies under high pressure this rigidity is removed and the tentacle begins to retract, but then divides spontaneously into 'minute spherical droplets'. According to Marsland and Brown¹⁰⁸ pressures up to 160 atmospheres cause a decrease in thickness and an increase in length of pseudopodia, and the sudden application of 250 atmospheres will cause an abrupt cessation of pseudopodial activity, but not retraction. From the ease with which internal granules may be centrifuged down it was inferred that there is a continuous decrease in protoplasmic viscosity with increase in pressure. As Marsland and Brown indicate, such movements of granules are not

necessarily accurate indications of relative protoplasmic viscosity, as there are many complicating factors present.

Sufficient pressure causes retraction of the cleavage furrow in developing eggs. With two species of *Arbacia* Marsland¹⁰⁷ found that no furrow will form at pressures greater than 350 atmospheres and that previously formed furrows recede at this pressure. At lower pressures the rate of impingement of the cleavage furrow on the division axis is reduced. On the other hand, a pressure of more than 800 atmospheres is required to block cleavage of the eggs of *Ascaris*.

Protoplasmic streaming velocity and viscosity are both reduced by pressure, and with *Elodea canadensis* streaming is abolished at 400–500 atmospheres.

Marsland has found that for each increment of 68 atmospheres in pressure a number of cell characteristics are diminished to 0.76 of their former value. This is true of the viscosity of two species of *Arbacia*, two species of *Amoeba*, and of the leaf cells of *Elodea*, the rate of impingement of the cleavage furrow on the division axis of two species of *Arbacia*, and the velocity of protoplasmic streaming in *Elodea*.

From these studies relatively detailed hypotheses have been advanced concerning the role of reversible gelation processes in cell division, protoplasmic streaming, and amoeboid movement.

It is clear that there is considerable complication in the mechanisms underlying these changes due to pressure. It is just possible, however, that the factor underlying inhibition of movement is in all cases the same—increased hydration of the protein molecules involved. There is evidence that muscular contraction, both of striped and of plain muscle, and the cleavage of cells, is associated with a decrease in solubility of long protein molecules.* According to Mirsky (1938) this change may also be produced *in vitro* by dehydration. We must therefore consider the possibility that one of the primary actions of high pressure is to prevent this dehydration and thus prevent movement.

These studies of the effect of pressure place a most important instrument in the hands of both large-scale and micro-biologists. It will be of the greatest interest to see, for example, whether cultured kidney tubules retain their secretory power under high pressure, and to see whether stomach tissue slices secrete HCl under high pressure; light may be thrown on other problems, such as the secretion of saliva by the salivary glands and the nature of the forces involved in the invagination of the blastula.

d. The Measurement of Volume Changes.

The study of volume changes has been useful mainly in connexion with permeability studies, though in other fields volume-change studies

* Mirsky^{112, 113, 114}; Bate Smith⁵; Danielli²⁸.

are a useful adjunct to other methods. The volume of a cell is a function of the number of osmotically active particles within it,* and change in the number of these particles (ions, sugar molecules, &c., colloids, &c.) brings about a change in the volume. The number of particles within the cell may be modified by passage through the cell membrane or as a result of activity inside the cell; this activity may involve either the synthesis of new molecules or changes in the state of aggregation of pre-existing molecules. The osmotic pressure of the external medium may, or may not, be important, according to the equilibrium conditions of the cell.

As examples of this we may take first the red cell. In NaCl solution the interior of the red cell contains a considerable amount of chloride ion. If the NaCl is replaced by an osmotic equivalent of Na_2SO_4 , chloride ion exchanges quantitatively for sulphate ion across the cell membrane. Then for each two Cl^- ions originally present there remains only one SO_4 ion, so that the number of osmotically active particles inside the cell is diminished: consequently the red cell shrinks on transference from NaCl to Na_2SO_4 . Furthermore, since the membrane has inappreciable structural rigidity and the cell has no active water-excretion mechanism, the red cell volume is a direct function of the external osmotic pressure; as this is lowered the cell volume increases, the cell eventually bursting. Ponder has shown that the volume changes are accompanied by a change from the discoid to the spherical form and the surface area of the red cell is unchanged during the swelling process. The light-transmitting power of a suspension of red cells is a function of the cell volume and so may be studied with a photo-electric cell or equivalent device. This method has been used by very many workers. Suitable apparatus for accurate work has been described by Jacobs^{83, 84} and by Ørskov¹²⁰.

The *Arbacia* egg, on the other hand, is relatively impermeable to crystalloid anions and undergoes little change in volume on transferring from NaCl to Na_2SO_4 . But it does swell in media of lower osmotic pressure. With this cell the surface area increases as the cell swells, finally rupturing in solutions having an osmotic pressure less than about 40 per cent. of that of sea-water. Similar swelling of both red cells and of *Arbacia* eggs occurs when the cells are transferred to a solution of a penetrating non-electrolyte, such as glycol. Many studies on *Arbacia* eggs have been made, especially by R. S. Lillie⁹⁸, by Lucké¹⁰², and by Jacobs and their colleagues⁸⁴.

Very different is the behaviour of the trout egg (Gray⁵²). The volume of this cell does not change with moderate variations in the salt content of the external medium, so long as the cell is alive. When the cell dies the volume changes in a manner rather similar to that of a block of

* Except in cases where increase in volume is restricted by the rigidity of an enveloping membrane, as with many bacteria and plant cells.

gelatin. This is due to the membrane becoming freely permeable to salts and water, but not to the yolk globulins, &c.

Swelling methods may also be applied to more complex organisms, e.g. Pantin's¹²² work on *Gunda ulvae*. Pantin found that this worm may swell to a remarkable extent without observable injury, provided a trace of Ca is present in the medium.

As a result of activity the osmotic pressure inside a muscle increases and the muscle swells in consequence. Other volume changes take place during activity. Meyerhof¹⁰⁰ has endeavoured to correlate these changes with the chemical changes occurring during activity.

iv. HEAT AND ELECTRICAL INVESTIGATIONS

a. Measurements of Heat Production.

Accurate measurements of the heat production of muscle and nerve have been made by Hill, Hartree, and their collaborators.

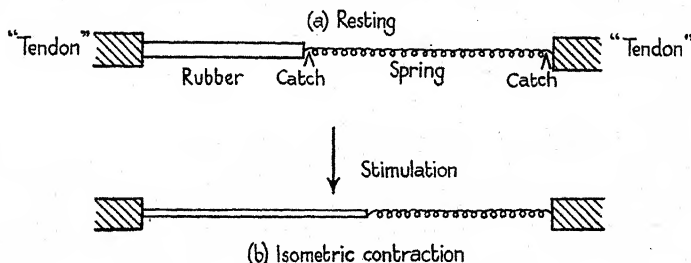
Many methods of measuring heat production are theoretically available, but of these the shielded thermopile method recently developed by Hill and Downing, using a fast galvanometer coupled with a photo-electric cell and a second galvanometer to obtain amplification of the thermopile current, provides by far the best technique yet developed.

The resting heat rate, both of muscle and of nerve, is practically constant.

The earlier measurements of Hartree and Hill⁶² showed that the heat evolution of a stimulated muscle occurs in three phases. The first phase involves a sudden evolution of heat occurring very shortly after stimulation, and complete before the maximum tension is reached. The second phase of heat evolution is closely associated with relaxation. The first and second phases may be complete in a fraction of a second. The third phase, which may last for many minutes, is probably due to the chemical processes associated with recovery and is not directly related to the contractile process.

Fenn⁴⁵ found that in an isometric contraction (response to stimulation without shortening) less energy is evolved than in a contraction in which the muscle does work by shortening against a load: i.e. in the latter case the heat evolved + the mechanical work done is greater than the heat evolved in the former case, when no work was done. This appeared to imply that a muscle 'knew' what load it was shortening against! and provided additional energy to compensate for it. Hartree and Hill⁶² found that, in a single twitch, the total energy set free is the same whether work is done or not; when mechanical work is done, the relaxation heat is diminished by an amount equal to the work. But, in agreement with Fenn, it was found that in a tetanic contraction extra energy is liberated if mechanical work is done.

The recent measurements of Hill⁷⁴ show that the ratio, total energy released : initial energy, is close to 2. For individual muscle preparations the ratio varied between 1.73 and 2.34. The value of the ratio for a given preparation was the same for either single contractions followed to complete recovery or in a long series of contractions in a steady state. During a steady state accumulation of the recovery heat occurs. During such a steady state the rate of recovery heat may be several times the resting heat production. The ratio, mean heat rate during steady re-



TEXT-FIG. 2. Diagram of muscle fibre (a) resting, (b) in isometric contraction.

covery : resting heat rate, varies in individual preparations from 3.8 to 7, i.e. the recovery heat rate lies between 2.8 and 6 times the resting heat rate.

A contracting muscle obeys the extremely simple relationship:

$$(P+a)V = b(P_0-P) \quad (1)$$

or
$$(P+a)(V+b) = b(P_0+a) = \text{constant.} \quad (2)$$

Here P = load lifted, P_0 = full isometric tension, V = velocity of shortening, b defines the absolute rate of energy liberation, and a is a term having the dimensions of force and defined by the relationship: extra heat* liberated during shortening = ax , where x = shortening (Hill⁷³). An active muscle may be regarded as a two-component system, consisting of (1) an undamped purely elastic element, in series with (2) a contractile element having equation (2) above as its characteristic equation. According to Hill there may also be viscous and viscous-elastic elements present, but in the case of striped frog's muscle they are relatively unimportant. A 'resting' frog's muscle may be regarded, to a first approximation, as behaving like a strip of rubber (element 1) tied to the end of a spring (element 2) which is stretched between two catches (Text-fig. 2). Stimulation releases the catches, enabling the spring to contract. Then in, for example, an isometric contraction, the rubber stretches as the spring contracts. In relaxation an unknown element is brought into action, stretching the 'spring' and allowing the 'rubber' to return to its original length. During contraction the

* i.e. heat liberated over and above that liberated in isometric contraction.

contractile element liberates heat which is contributed to the first heat production, and during relaxation the shortening of the elastic element is accompanied by the conversion of its potential energy into heat, which is contributed to the relaxation heat.

The heat production of nerve in response to stimulation is very much less than that of muscle and occurs in two phases: an initial phase accompanying the response to a stimulus; and a second phase of long duration, probably connected with the chemical events of recovery, in which the heat evolution is 10 or more times greater than the initial heat. For the non-medullated limb nerve of the crab the initial heat is 2×10^{-2} ergs per sq. cm. of nerve axon surface: for the medullated nerve of the frog it lies between 5×10^{-3} and 2.5×10^{-4} ergs per sq. cm., according to the experimental conditions (Hill⁷²). As Hill has pointed out, this is a remarkably small amount of energy, even for a surface phenomenon; for example, the free surface energy of the olive oil-water interface is about 20 ergs per sq. cm. and of cell surfaces is about 0.05 to 1.0 ergs per sq. cm. It is therefore very improbable that passage of the impulse involves more than a small physical change in the nerve plasma membrane; such changes might be considered as changes in orientation of the lipoids of the membrane. Complete breakdown of the membrane is very unlikely.

The heat production of the eggs of *Arbacia punctulata* has been studied by Rogers and Cole^{124a}. The heat production of the resting unfertilized eggs is comparatively small. On fertilization the rate of heat production leaps up to a value as much as 12 times greater than the previous rate. This increase is only partially sustained, the rate falling to about two-thirds of this value (6.8 times the original value) after 20 minutes, and remains constant for the next 30 minutes. A further fall then occurs to a value 5-6 times the original value, and this remains constant up to the eight-cell stage.

b. Electrical Resistance and Capacity (Impedance) Studies.

By the use of alternating current of varying frequencies the apparent resistance and capacity of biological systems can be obtained. Outstanding work is that of Höber^{77,78} on the red cell, of Fricke^{48,49,50} and his colleagues on the red cell, yeast cells, and leucocytes, and of Cole³⁰ and his colleagues on marine ova, *Nitella*, and on the giant nerve axons of the squid.

A general conclusion emerges from the work on the various cells; the cell plasma membrane has a capacity of the order of one microfarad per sq. cm. and a resistance of the order of 10^4 ohms. The capacity is very high indeed and the resistance, being that of a membrane only 10^{-6} - 10^{-7} cm. in thickness, corresponds to a specific resistance of 10^{10} - 10^{11} ohms, a value similar to that of olive oil. It is remarkable that the wide variety

of cells studied should all conform to this pattern. The action of various ions on the resistance and capacity has as yet been studied but little, but Blinks^{12,13} has shown that KCl greatly lowers the resistance of *Nitella* membranes. On the other hand, neither KCl nor CaCl_2 has much effect upon the resistance of the medullated sheath of frog nerve (Danielli⁴⁰). There is little doubt that the chief function of the sheath is to act as an electrical insulator,* so that it is advantageous to have the resistance of this membrane insensitive to ions.

A further general conclusion is that the high resistance of cells to direct current is almost entirely due to the cell membranes. By using high-frequency alternating current it is found that the resistance of the interior of, for example, marine cells in sea-water is comparatively low, rarely greater than 4 times that of sea-water, although the cells may be highly resistant to direct current. Similarly the resistance of the cytoplasm of the body cells of mammals and other land animals is not vastly different from that of the same animals' plasma.

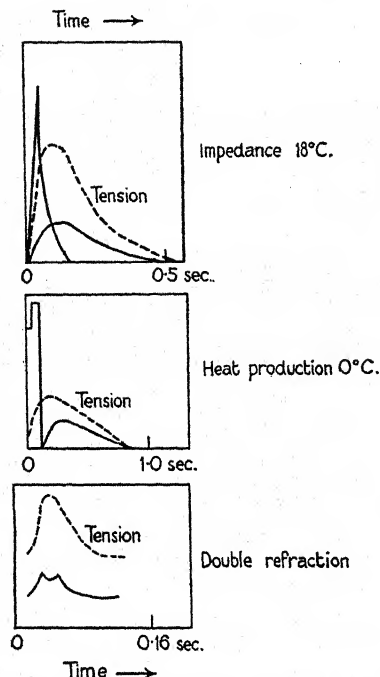
Generally speaking, in other respects the results of resistance and capacity measurements have so far been disappointing, as the method seems to be insensitive to the minor changes in membrane properties accompanying normal cellular activity and in some cases, where membranes are known to be grossly damaged, no change in resistance or capacity appears as a result of the damage. Some important results have been obtained on eggs, muscle, and nerve. Changes in the resistance and capacity of some marine eggs occur as a result of fertilization. Hubbard and Rothschild⁸¹ have found peculiar rhythmical changes in the impedance of trout eggs, the significance of which is obscure. Dubuissou⁴³ has made a successful study of impedance changes in frog muscle. In a strictly isometric muscle there are two waves of increase in impedance following the stimulus. The first of these probably begins before the contraction and attains its maximum during the phase of rising tension. The second reaches a maximum during relaxation and returns rather slowly to the base-line: this second change in impedance remains permanently in muscles poisoned with iodo-acetate. Dubuissou suggests that the first change is related to the transformations of adenylyl pyrophosphate and the second change to the transformations of phosphagen. Text-fig. 3 shows some of the various physical changes known to take place in a stimulated muscle. The experiments for the different measurements were made at different temperatures, so that comparison must be referred to similar points on the tension curve. If this is done it will be seen that the two phases of the initial heat production and the two phases of diminution of double refraction occur at roughly the same

* Katz and Schmidt⁸⁶ have shown that an impulse passing down one fibre in a bundle of non-medullated fibres is able to change the excitability of adjacent fibres.

points on the tension curve. We have already referred to the fact that the detailed studies of Meyerhof and his colleagues have shown that the muscle volume diminishes during contraction. Muralt^{116a} also found that the translucency of muscle diminishes during contraction. Dubuissou

discusses the parallelism between the variation of these physical properties in tetanized muscle and in muscle in anaerobic conditions or when poisoned with iodo-acetate.

Perhaps the most important work so far accomplished by impedance measurement is that of Curtis and Cole on the change accompanying the conduction of the impulse in nerve and large plant cells. R. S. Lillie⁹⁷ has summarized and discussed the evidence for the view, put forward in 1909 and in 1912 by Bernstein¹¹, that the basic phenomenon in response to a stimulus is a transient increase in permeability to ions, and in the case of nerve, the transmission of the impulse consists essentially in the travelling of this increase in permeability along the axon membrane, together with the concomitant discharge of ions through the permeable area which is reflected in the action potential.



TEXT-FIG. 3. Physical changes taking place in stimulated muscle.

Using a very refined technique, Curtis and Cole^{34, 35} were able to show that, with *Nitella* and with the giant axons of the squid, this transient increase in permeability (or conductivity) does in fact accompany the action potential. The conductivity of the plasma membrane is increased about 200-fold. The capacity changes comparatively little.

Large though the change in conductivity is, the residual resistance is still comparatively high, so that it is evident that the membrane does not break down entirely during the transmission of the impulse. This confirms the view of A. V. Hill, based on heat-production measurements, that complete breakdown was improbable.

c. Potential Measurements.

A great variety of tissues and cells have been used for potential measurements and such measurements have played a vital role in, for example, the investigation of the transmission of the nerve impulse.

The results of these investigations are too well known to need recapitulation here. We may, however, consider for a moment the interpretation of potential measurements. On this subject Gasser⁵¹ bluntly remarked: 'It would be to the advantage of every electro-physiologist to nail this notice on an imaginary laboratory door—you cannot determine a process from a potential.' There has been no change in the accuracy of this remark. Students of electro-physiology are therefore increasingly obliged to undertake other physical measurements, running parallel to potential measurements. But this work is still in its infancy. Consequently little more can be said of the resting (injury) potential than that it behaves as though it were a K^+ diffusion potential arising across the plasma membrane, and that the plasma membrane of many cells is more permeable to K^+ than to other cations such as Na^+ and Ca^+ . The potential is to some extent a function of metabolism, but probably rather indirectly so. These conclusions are based on the work of MacDonald¹⁰⁴, Höber^{76, 77}, Osterhout¹²¹, Cowan³³, and many others. The biological studies and the mechanisms by which potentials arise have been reviewed by Davson and Danielli (1941)⁴².

The action potential of nerve, muscle, and of large plant cells is now believed by almost all workers to involve a discharge of the resting potential as a result of increased permeability of the plasma membrane. As mentioned before, there is a large transient increase in permeability now known to occur during transmission of the impulse in *Nitella* and in squid nerve. It is known, too, from the work of Katz⁸⁵ and of Hodgkin⁷⁹ that in electrical stimulation of nerve, whereas very weak stimuli cause no local response, stimuli a little below threshold cause a local response, so that this change in permeability is probably capable of gradation and is not necessarily explosive (all-or-none) in character.

Satisfactory analysis of more complicated systems, such as frog-skin, has only just begun.

d. *Electrophoresis Measurements.*

By measuring the rate at which a cell moves in a potential gradient it is possible to calculate the average charge density—that is, the number of ions per sq. cm.—on the cell surface, or on the surface of the outermost membrane of the cell. In solutions more dilute than about 0.1 N to 0.001 N, according to the cell, the results obtained are unreliable; the charge on the cell surface leads to the local concentration of ions near the surface being much greater than the average, and in consequence of this the cell is exposed to a lower potential gradient than the average value given by the recording instruments. Consequently many of the results in the literature showing a dependence of surface charge on salt concentration must be regarded with suspicion.

Hartley and Roe⁵⁸ have shown that the concentration of ions near

a surface may be calculated from the electrophoretic mobility, and Danielli^{37,41} has shown that this concentration may be determined also from the charge density, using the theory of Debye and Hückel to calculate the volume in which the surface ions are distributed. The concentration of ions at a surface may be several hundred times that in the surrounding bulk salt solution. The average concentration at the surface of cells is usually 1.3 to 5 times greater; this involves, for example, a pH difference between the salt solution and the cell surface of between 0.1 and 0.7 pH units. It is possible that in localized regions of the cell surface the excess of ions is much greater.

Abramson's book,¹⁷ *Electrophoretic Phenomena*, contains a survey of measurements made on cells.

*e. pH Measurements.**

pH measurements made by vital staining dyes and by the injection of dyes into cells have already been referred to under micro-dissection. Three methods have been used to study pH changes due to activity. (1) The evolution of CO₂. This is complicated by diffusion factors, so that it cannot be used for studying the rapid changes in pH which occur during, for example, the contraction of a muscle, but it is the best method for studying recovery or other slow processes. It has been used successfully by D. K. Hill⁷⁵ to investigate the recovery of frog muscle after stimulation. (2) Colour changes with indicators. This is relatively insensitive unless roughly monochromatic light and a good photo-electric cell are available, or better still, if spectro-photometric studies can be made. Margaria and Pulcher¹⁰⁵ have used the method for studying changes in muscle. The technique would probably pay for further development. (3) The glass electrode. This method has been used particularly successfully by Dubuisson⁴⁴. It is not available for studying recovery processes, but does enable rapid changes to be studied with some accuracy, subject to an unknown error due to the time taken for acid generated within the cells to reach equilibrium with the fluid bathing the cells.

According to Dubuisson there are four changes in pH taking place in smooth muscle during the contraction-relaxation cycle, and probably the same changes also take place in striped muscle, though the greater speed of the contraction makes it difficult to secure adequate results for striped muscle. These changes in smooth muscle are:

- (a) Alkaline; probably precontractile; of the order of 1 mm.³ or less of CO₂ per gramme of muscle per twitch; cause unknown.
- (b) Acid; probably occurs early in the contractile process; of the order of 3 mm.³ CO₂ per gramme; cause possibly hydrolysis of adenyl pyrophosphate.

* See also under 'Micro-dissection'.

- (c) Alkaline; has its maximum at the beginning of relaxation; of the order of 1 mm.³ CO₂ per gramme; cause possibly hydrolysis of phosphagen.
- (d) Acid; begins at maximum tension, but only reaches a maximum several minutes later (at 20° C.); cause probably lactic acid formation.

Dubuisson, assuming that the pH changes (b), (c), and (d) have the chemical basis given above, has calculated the form of the heat production of smooth muscle, assuming it to be exclusively due to the three reactions mentioned. The anaerobic heat should begin with a positive heat in the early stages of contraction, followed by a second positive heat production near the maximum and during relaxation (early stages) and by a negative heat in the later stages of relaxation. Finally there is a prolonged positive recovery heat, lasting many minutes, due to the lactic acid formation. This calculated anaerobic heat resembles in many ways the anaerobic heat production of muscle found by Hartree. Dubuisson makes it clear that these theories must be regarded with reserve.

D. K. Hill, measuring the volume of CO₂ produced at 0° C. by the frog sartorius muscle, finds that, under circumstances in which lactic acid is not formed, only alkaline changes associated with the hydrolysis and resynthesis of phosphagen are observed. When lactic acid production is permitted, an acidity is found which may (e.g. with cyanide poisoning) take 15 minutes to become half complete.

Many interesting variations in these pH changes are found when enzyme poisons such as iodo-acetate, cyanide, and azide are used, which are assisting greatly in allocating the relative positions in time of the various chemical reactions known to accompany or succeed contraction.

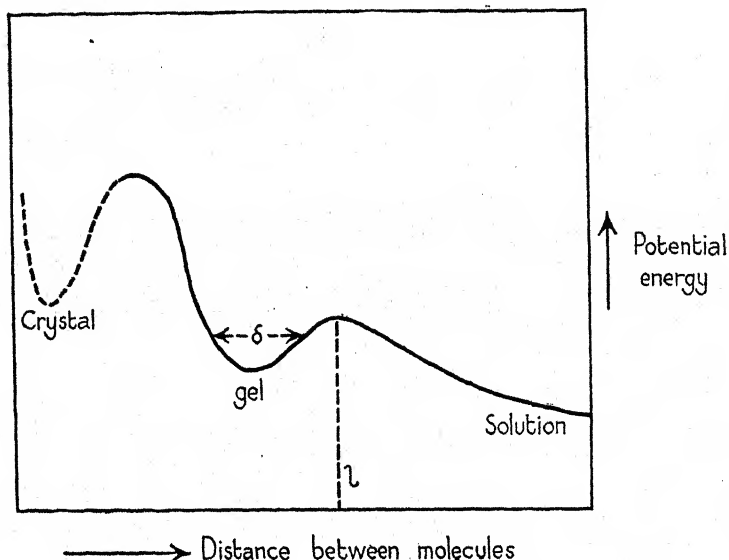
V. MODELS OF BIOLOGICAL SYSTEMS

Owing to the great complexity of living systems, those working on physical properties of cells must frequently resort to a more or less crude model. Until recently these have been largely composed of iron wire, gelatin, nitrocellulose, and olive-oil emulsions. Recently more suitable models have become available, due to the development of surface and colloid chemistry. These models enable us to test the feasibility of some theories of biological mechanisms with materials which, though not identical with natural membranes and colloids, nevertheless very closely resemble them in many important features, such as the nature of the molecules involved and the physical dimensions of the structures considered. The models which will be particularly considered are: (a) the tactoid as a model of plasmagel; (b) lipo-protein films and monolayers

as models of the plasma membrane and of other biologically important surfaces.

a. Tactoids.

If a solution of tobacco mosaic virus is left standing under suitable conditions, it separates into two or three compounds: (a) crystals of the



TEXT-FIG. 4. Potential energy of colloidal particle as a function of the distance between adjacent particles (tactoid-forming type of particle).

virus; (b) a top layer which has no rigidity, but which shows double refraction on being made to flow; (c) a denser lower thixotropic gel layer having some rigidity and which is spontaneously double refracting. During the separation of the two layers needle- or spindle-shaped bodies of higher or lower density form in the body of the solution, either rising or falling slowly according to their density. Coalescence of these bodies gives rise to the two layers. Bernal and Fankuchen¹⁰ and Bawden *et al.*⁶ have shown by X-ray studies that the lower layer consists of a hexagonal lattice in which the needle-shaped protein particles are oriented parallel to one another, the distance between adjacent particles varying between 50 m μ and 12.5 m μ as the concentration of protein increases. In the crystal the molecules are much closer together than this.

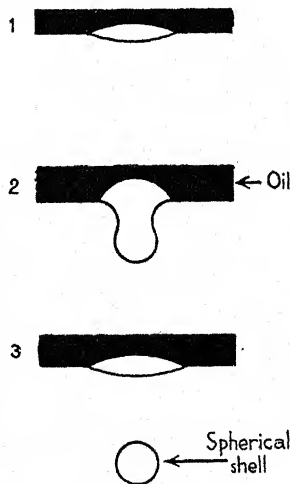
From these observations it follows that there must be at least two regions, (1) the crystal, and (2) the gel, in which the forces operating between adjacent protein molecules may give rise to a stable or metastable configuration in which the protein molecules have a definite orientation with respect to one another, and that the intermediate

regions are positions of relative instability. Recently Langmuir⁹² and Levene⁹⁴ have shown on theoretical grounds that the electrostatic forces operating between colloidal particles may lead to the potential energy of a particle being less at large distances of separation and over a small range δ of separation distances, than over an intermediate range of distances. The separation distance δ , though relatively small, is much greater than the particle separation in the crystal. Thus a solution of composition corresponding to l (Text-fig. 4) should separate spontaneously into two phases, one more concentrated, the other less concentrated. The more concentrated phase, corresponding to separation distance δ , corresponds to the spontaneously doubly refracting virus gel.

These theoretical and experimental studies lay the foundations for the investigation of the rigid gel-like spontaneously doubly refracting bodies which are known to play such a large part in protoplasmic movement. The investigation of the influence of particle size, charge, and hydration on such gels may throw much light on the mechanism of movement.

b. Films and Monolayers.

Since the work of Overton* many workers have believed that the cell plasma membrane is a lipid layer, and we know now that, owing to the great surface activity of proteins, if it is a lipid layer then it must have a layer of protein adsorbed on its two surfaces. To test the lipid layer hypothesis Danielli constructed some artificial thin lipid layers, stabilized by adsorbed protein, by placing an oil layer above an aqueous layer and running drops of water into the oil layer. The drops settle down at the oil-water interface with a thin film of oil between the two aqueous phases. If the density of the aqueous solution in the drop is greater than that of the aqueous layer, part of the drop may fall away from the interface and enter the lower aqueous layer, coated by a complete shell of oil (Text-fig. 5). Such spherical shells are relatively stable, so that we can say with confidence that the lipid-protein paucimolecular layer theory of the cell membrane is quite feasible.



TEXT-FIG. 5. Formation of a spherical shell of oil.

Physical studies on such films, especially those not exceeding $5\text{ m}\mu$ in thickness, may reveal many facts important for biology. They may

* See Chapter III.

differ from thicker oil layers, in so far that in a layer only $5\text{ m}\mu$ thick a large proportion of the membrane molecules will be oriented at the two membrane-water interfaces, whereas with thicker layers this will not be the case. At present not all the experimental difficulties have been surmounted and the greater part of the information available on films comes from the study of monolayers at the air-water and oil-water interfaces.

CHAPTER II (cont.)

PHYSICAL AND PHYSICOCHEMICAL STUDIES OF CELLS

PART II. MONOLAYER TECHNIQUE

By J. H. SCHULMAN

i. INTRODUCTION

THIS technique is a simple and convenient way of studying:

- (1) The structure and chemical composition of interfaces.
- (2) Chemical reactions and molecular associations taking place at interfaces. The interfaces which have special biological interest are those between a monolayer and its substrate as envisaged by the air/water interface and the lipoid or oil/water interface.
- (3) From a knowledge of the chemistry and behaviour of molecules of biological importance such as lipoids and proteins at oil/water interfaces direct analogies have been drawn as to their possible function *in vivo*. This has also been especially applied for those substances which have marked biological effects in minute concentrations and show a puzzling specificity. It has been shown that an identical specificity exists between the action of these highly active substances on a synthetic cell surface as understood by mixed lipo-protein monolayers and their action on cells such as red cells, unicellular animals, or bacteria, &c.

ii. METHODS

Literature: An excellent detailed survey of all this field has been given by J. S. Mitchel⁴ in the colloidal properties of the cell. Details of the methods used in the monolayer technique with full literature references are given by J. H. Schulman⁶ and by N. K. Adam¹.

iii. PROPERTIES OF MONOLAYERS

a. *Electrical Properties.*

The deposition of a monolayer of molecules at the air/water or oil/water interface changes the surface potential. This potential is that measured between an air electrode and the surface of the aqueous solution and, as will be shown later, is related in some way to the vertical component of the resultant dipole moment of the polar groups of the molecules forming the monolayer.

The most surprising and useful fact regarding this potential change is that it is constant with time. All potentials of this description should

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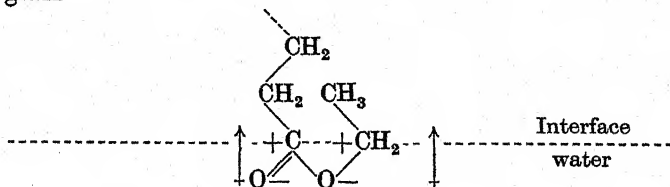
be polarized by the ions of the opposite sign in the aqueous solution or should decay with time according to the capacity and resistance of the system.

O. Gatty⁶ suggested the following explanation for this phenomenon: the electrical double layer composed of sheets of positive and negative charges would be partially neutralized from the aqueous side of the electric double layer by ions in the solution. No polarization can take place from the air side, since any ions in the air which would become attracted to the surface to build up the neutralizing layer would be sucked continually into the solution by their own mirror image forces.

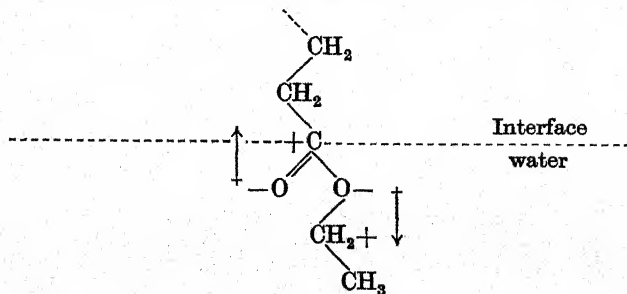
Indeed it can be demonstrated by a suitable choice of a non-aqueous medium to replace the air, in which ions can be dissolved, that potentials of this description at an aqueous interface do decay with time according to the usual theory.

Strong indication that this surface potential is related to the resultant vertical component of the various polar groups in the molecule of the film-forming substance can be shown by the case of the aliphatic esters. Films composed of esters consisting of a long hydrocarbon chain attached to the acid radical and a short hydrocarbon chain attached to the alcohol radical such as ethyl myristate orientate themselves when the film is expanded with the two chains side by side. On compression of this film the short chain is reorientated into a position opposite the long hydrocarbon chain, thus changing the resultant electrical field of the alcohol radicle dipole.

In the initial position the resultant dipole is large, as can be seen from the diagram



and is experimentally found to be 5.10^{-19} E.S.U., in the compressed position the molecule orientates similar to the second diagram



and the resultant dipole moment as measured by surface potentials is very small, and actually in the case of cetyl palmitate is zero.

There are many other examples showing that the surface potential is related in some direct way to the polar group of the film-forming molecule. The ionization of a fatty acid film as the hydrogen-ion concentration changes in the underlying solution gives radical changes in the surface potential, starting with more than +400 mV in strong acid solutions to -150 mV in strong alkaline solutions. The surface potential technique therefore becomes a very useful and simple tool for investigating chemical reactions and associations which a monolayer may undergo with ions or chemical substances present or injected into the underlying solution.

Examples of these reactions will be given later together with their biological analogies. Any change in the polar group of the molecule either by reorientation, association, or in its chemistry is immediately reflected in the surface potential.

The surface potential change is given as $\Delta V = 4\pi n\mu$, where n is the number of molecules per sq. cm. at the interface and μ is a function of the polar group or groups of the film-forming molecule.

The surface potential is measured by means of an air electrode in the shape of a thin wire on which is deposited some radium D or polonium, or a milligram of mesothorium in a tube placed in the near vicinity of the wire. These substances ionize the air gap of a few millimetres between the end of the wire and the aqueous surface. The air electrode is connected through an earthing switch to an electrometer or to a valve amplifier with a galvanometer attached. The electrometers or galvanometers are used conveniently as null instruments. This side of the circuit must be well insulated and shielded against electrical disturbances. The aqueous solution is connected to earth by means of a calomel half cell, with salt bridge or simply by means of a platinum or silver wire. Between the half cell and earth a potentiometer is arranged so that an opposite potential can be placed across the air gap to bring the electrometer needle to zero. The change of this potential due to the deposition of a monolayer is termed the surface potential of the film. The number of molecules per sq. cm. being known, μ can be plotted in electrostatic units against the area of the molecule.

The air electrode can be so manipulated as to move over the whole surface of the interface, thus establishing the area per molecule in which electrical homogeneity occurs. These areas usually denote changes of state of the film.

b. Surface Pressures

If the monolayer consisting of water-insoluble molecules be deposited on to the surface of an aqueous solution contained in a trough filled to

a bevelled edge, and the area of the film is restricted between a waxed slide and a movable waxed boom which can be kept in its original position by means of a torsion wire, then the surface pressure is defined as the force per cm. of the boom which is required to hold the boom in position. Leakage of the film past the boom is prevented by means of vaselined silk threads or thin platinum ribbons.

This surface pressure (F) is plotted against the area per molecule (A) or the number of molecules per sq. cm. (N).

If the surface tension of a free-water surface is γ_0 and the surface tension of the surface after deposition of a monolayer is γ_m , then the surface pressure is equal to $\gamma_0 - \gamma_m$.

This form of trough with surface boom is usually called a Langmuir-Adam trough, after the names of the two chief workers in this field. It is useful to look upon the physical characteristics of a monolayer as measured by the Langmuir-Adam trough in their three-dimensional analogies.

Thus since the insoluble molecules are restricted to one side of a barrier and their pressure measured against the free-water surface, it has all the features of a two-dimensional osmotic pressure. The barrier being semi-permeable restricts the monolayer molecules and permits the diffusion of water molecules. If the adhesion between the molecules in the film is small under the usual physical conditions (T , F , A) then they will obey the gas law and one may write an equation of state $(F - F_0)(A - A_0) = KT$ similar to the three-dimensional gas law.

The insoluble molecules forming the monolayer consist of a polar portion and a non-polar portion. The polar portion will be attracted into the water phase and the non-polar portion will be repelled by the water. Therefore one may imagine the film as a double or duplex film, F_0 being related to the lateral adhesion between the non-polar portions and A_0 related to the area of the polar portions of the molecule.

It is actually the mutual associating forces between the polar and non-polar portions of large molecules which are the interesting characteristics of the molecules at interfaces, since it is only due to adlineation of the molecules by the asymmetrical field of forces at an interface which brings these forces into action.

In bulk solutions these forces can only be determined by viscosity and micelle formation. The energy of association between molecules when they are adlineated at an interface can be of the order of 30,000 cal., which is more than sufficient to change the chemical and, more important, the biological activity of the compound. The importance of orientation in relation to activity of the molecules will be stressed later.

As soon as the energy of the associating forces between the molecules commences to predominate over their kinetic energy, the monolayer

will change its state. In fact the force area curve with temperature change gives a complete phase diagram for a monolayer.

The various kinks in the curves are transformation points for the various states. These points have been given direct three-dimensional analogies; thus the area per molecule for the commencement of the liquid state is equal to the $(\text{volume})^{2/3}$ for a molecule in the interior of a drop of the liquid. The area per molecule at the end of the liquid state is the area per molecule which exists at the surface of a drop of the liquid; or the monolayer at its greatest compression in the liquid state is equivalent to a very thin drop of one molecule in thickness.

Complete adlineation of the molecule is required before the molecule can exist in the solid state in a monolayer.

The sharp changes and limiting areas in the force area curves are related therefore to the chemistry of the non-polar portion and to the nature of the polar group, and it is reasonably easy to distinguish from the nature of the curves the possible structure of the film-forming molecule (see example later).

It is experimentally possible to measure in a simple manner changes in surface pressure from 60 dynes/cm. to 0.001 dynes/cm.; this is equivalent in three dimensions to measuring the physical characteristics of a compound from about 100 atmospheres to a one-thousandth of atmosphere.

c. Surface Viscosity.

Owing to the high degree of orientation of the molecules at an interface very high surface viscosities are obtained for surface films. A knowledge of the change in surface viscosity with area per molecule gives useful information on the structure and associating properties of the compounds which form monolayers. This is especially important for protein films. Two standard methods are used which give results for viscosity changes, but information on the absolute viscosity is difficult to obtain, and the theory is still being developed.

One method is to measure the time necessary to flow a monolayer through a narrow canal under a weak pressure gradient. A further method is to oscillate a vane in the surface and measure the logarithmic decrement of the successive oscillation produced by the clean surface and the surface covered by the film.

d. Multilayer Formation.

Information regarding the area and associating properties of molecules can be obtained from the three described techniques, but it is difficult to determine the thickness of the layer or the vertical dimension of the molecule forming the monolayer. The multilayer technique is a simple way of determining this factor. If a slide which has a hydrophobic

surface or is made hydrophobic by the deposition of the first monolayer is pushed through the surface of the aqueous solution upon which a monolayer is kept at constant surface pressure, independent of the area of the monolayer, then under certain conditions the monolayer will be deposited on to the slide.

The physical condition necessary for deposition of the monolayer is primarily that of the contact angle which the covered aqueous solution makes with the penetrating slide. This contact angle can be readily controlled by changing the surface pressure of the film and also by varying the chemistry of the polar group of the film-forming molecule.

The film can only be deposited on the slide if the contact angle is obtuse; in general at low surface pressures this angle is obtuse only on the downward movement of the slide through the surface of the aqueous solution and not on the upward movement. Hence deposition in this case will occur on the downward movement and is termed *X* deposition. At high surface pressures the contact angle is obtuse both on the downward and upward movement of the slide through the surface, the film being deposited on both movements. This deposition is termed *Y* deposition. On repeated movement of the slide through the surface the films will be rolled on to the surface of the slide according to the above conditions. The surface pressure of the film on the aqueous solution is conveniently kept constant by so-called piston oils. Lenses of these oils such as oleic acid (30 dynes/cm.), triolein (21 dynes/cm.), castor oil (16 dynes/cm.), tritolyl phosphate (10 dynes/cm.), keep a constant surface pressure with the film of these substances irrespective of the area of the film so long as there is excess of the oil on the surface in the nature of a lens. The depositing film and the pressure film are kept separated by a vaselined silk thread.

The molecules in the consecutive layers will orientate themselves irrespective of the method of deposition into double-layer or single-layer lattices according to their crystal structure.

The built-up multilayers are half-way between an amorphous mass and full crystallization, the molecules in the consecutive layers having only one common axis, that normal to the surface. This orientation holds throughout the whole thickness of the multilayer, even up to a 1,000 layers. As soon as the thickness of the multilayer is equal to a quarter wave-length of light, Newtonian colours set in when they are seen in polarized light at a low angle of incidence. This is a convenient way of measuring their thickness; the colours when viewed in white light, or their intensity when viewed in monochromatic light, are sensitive to changes of only one molecule in thickness.

Since the polar groups in molecules give clusters of atoms heavier than the CH_2 group at definite spaced intervals, an X-ray beam will be reflected from these planes as in a Bragg diagram obtained from an

ordinary crystal. These main spacings obtained by permitting a monochromatic X-ray beam to travel along the multilayer at small angles of incidence give the thickness of the layer in the multilayer and the tilt of the molecule. Under careful X-ray examination of a thick multilayer the side spacings between the molecules can also be determined.

Substances which do not crystallize in bulk but only form greases can be made into two-dimensional crystals as in monolayers. These monolayers after being built into multilayers will give an X-ray diagram showing all the atomic spacings in the molecule. An X-ray diagram of a multilayer taken at one angle of incidence gives a pattern identical with a rotation picture of the crystal of the substance.

The value of this technique in determining the structure of a non-crystallizing substance obtainable only in minute quantities in combination with the other three surface techniques will now be demonstrated.

e. Determination of the possible Structure of the Tubercular Toxin Phthoic Acid.

Dr. Anderson (New York) isolated out of tubercular bacteria a small quantity of phthoic acid in the form of a grease. This acid possessed all the biological properties associated with the tubercular toxin. It was considered to be a fatty acid with twenty-six carbons and containing one optically active carbon. The carbons were tentatively supposed to be in the form of a C_{18} straight chain with a methyl group substituted alternatively in the CH_2 groups up the chain. A minute quantity of this grease was sent to E. Stenhagen at Cambridge, who found by using the described monolayer techniques with 0.3 mg. of material the following dimensions and electric properties of the molecule.

The area per molecule of a condensed film was 40 \AA^2 , the surface potential at this area was 200 mV, and the maximum length of the molecule was equivalent to 12 carbon atoms as illustrated by X-ray pictures of a multilayer of the substance.

The surface properties of the previously described formula are 30 \AA^2 , 400 mV, and 18 carbon atoms length of molecule.

A methyl group substituted in the α -position only increases the area per molecule of a straight hydrocarbon chain from 20 \AA^2 to 30 \AA^2 in a condensed film, leaving the surface potential at 400 mV. A double hydrocarbon chain increases the area to 40 \AA^2 , still leaving the surface potential at 400 mV. But a tri-substituted acetic acid with two long chains and a methyl group substituted in the α -position put the surface potential down to the required 200 mV. Thus the formula worked out from the monolayer techniques suggested a tri-substituted acetic acid with two long hydrocarbon chains of 10 and 12 carbons respectively and an ethyl group. Professor Sir Robert Robinson at Oxford was

attempting the synthesis of such a compound and found that the tri-substituted acetic acid compounds were biologically active whereas the di-substituted were inactive. The work at this stage has been temporarily suspended.

iv. THE STRUCTURE OF PROTEIN AND LIPO-PROTEIN FILMS.^{3,5}

a. Protein Films.

It is an astonishing property of proteins in general that solutions or solid particles of them spread at the air/water or oil/water interface to form a very thin film. This film is insoluble, except under certain conditions, in the underlying solution, although the protein itself is very soluble in the underlying solution.

Furthermore, these films upon compression develop very high viscosities and ultimately become so-called two-dimensional gels of varying rigidity and elasticity.

The suggested explanation for the formation of films of this type is that the globular protein molecule in solution unfolds at the interface owing to the asymmetrical forces. This permits the non-polar side chains to orientate and associate together away from the side chains with polar groups, leaving the backbone composed of the —CHR—CO—NH— chain separating them.

The picture created at the interface is thus of a triplex layer with non-polar chains in the air or oil phase, the backbone chain with the polar side chain drawn into the aqueous phase. Association by short-range forces between these three groups either intra- or inter-molecularly is probably responsible for the large molecule remaining at the interface and not dissolving away into the underlying solution.

Adlineation between the large molecules lying on the surface at high surface compression is probably responsible for the formation of the gels and films of very high viscosity. As can be expected, the rate of spreading of proteins and the properties of the film is dependent on the hydrogen ion and salt concentration of the underlying solution and on the chemical nature of the proteins. But in general a film of a protein becomes coherent by surface potential measurements and exerts a surface pressure of 0.2 dyne/cm. at a weight of 1 mg. per sq. metre.

At this area the films are fluid and on compression they gel and start showing strains in the film in the form of striations at about a third of this area.

This force area curve is reversible except when the film is held for any length of time at high pressures. High surface pressures force the film molecules into solution again. Whether only the smaller molecules in the film as breakdown products of the large protein molecule or

whether the whole film can be redissolved by these surface pressures is still a matter for research.

A highly compressed film showing striations is completely irreversible. The striations are micro-fibres with a hydrophobic coating, and they can actually be spun off the surface of the water into insoluble and non-spreading threads.

Proteins from the solid spread least of all at their iso-electric point, but a film spread at another pH is quite stable at its iso-electric.

Proteins from solution only spread better at their iso-electric in an artificial manner, since in comparison with solutions of other pH's they are least soluble and the protein appears at the surface from diffusion and solubility considerations quicker in an iso-electric solution than in a solution in which they are readily soluble. It is possible to overcome all spreading difficulties by dissolving the protein in suitable salts in the presence of iso-propyl alcohol. The salt and the alcohol spread the protein in a manner similar to the spreading of stearic acid with benzene, &c. The association between the molecules is broken down by dispersion and the alcohol artificially spreads the molecules by reason of its own spreading forces.

These solutions of proteins spread to films independent of the nature of the underlying solution. The protein molecule at the interface is distorted by reason of the separation of the non-polar side chains from the polar side chains. The continuation of the fundamental unit of the backbone $\text{CHR}-\text{CO}-\text{NH}-$ is further complicated by the presence of proline derivatives. These factors would necessitate the protein backbone to form loops instead of a continuous zigzag or any of the theoretical pictures of the α and β keratin type.

On compression of the film these loops would be forced out of the surface and are possibly responsible for the formation of the striations in the film in the form of hydrophobic folds. They are also responsible for the fact that the repeat unit $\text{CHR}-\text{CO}-\text{NH}$ can be compressed to 10 \AA^2 in the film which is well below the theoretical area possible of 15.7 \AA^2 .

Adlineation or disorder of the long molecules extended on the surface at low concentration or surface pressures possibly gives rise to the different types of force area curves found by many workers in this field. Indeed there is evidence from low pressure and surface viscosity work for these two types of films, according to whether time is permitted for adlineation to take place. It is possible by measuring very low surface-pressure area curves to determine the molecular weight of the protein molecules. The formation of gel films of a reproducible nature seems to be the characteristic of substances of high molecular weight at an interface, since the esters of polysaccharides or synthetic resins form films very similar to the proteins.

b. Lipo-protein Films.^{3,4}

In view of the fact that certain proteins such as gliadin are soluble in 60 per cent. alcohol and that most proteins can be dispersed in salts and iso-propyl alcohol solutions, lipoids can be added to the protein solutions. Mixed lipo-protein films can therefore be prepared with varying proportions of lipid to protein.

Cholesterol and gliadin mixed films have been made in this manner, and they show very interesting properties in relation to their surface viscosity. A gliadin film on a buffer solution of pH 7.4 becomes coherent at about 1 mg./m.² and commences to gel strongly at about 16 dynes/cm. On further compression the gel becomes more rigid until the irreversible film with striations appears.

Incorporation of 20 per cent. cholesterol to the gliadin has a striking effect on the force area curve. At 16 dynes/cm. the mixed film gels as before, the gelation becoming more rigid on compression until at about 21 dynes/cm. the rigid film suddenly liquefies. This liquefaction on compression and solidification of the film on expansion is a reversible process. The compressed liquefied film has all the properties and characteristics of a cholesterol film.

It could thus be supposed that at 21 dynes the cholesterol molecules in the monolayer of protein push the protein molecules into the underlying solution in the form of a double layer. On release of the pressure the associating short-range forces between the polar group and non-polar portion of the cholesterol molecule and the similar groups of the protein molecule permit the protein molecule to penetrate into the cholesterol upper film and thus re-establish the mixed monolayer. Other examples of penetration and adsorption of molecules into and on to monolayers will be given in the next section.

It has been shown that this sudden liquefaction phenomenon on compression of a mixed lipo-protein film is related to the salt concentration and pH of the underlying solution and also to the lipid protein ratio. It was found that the greatest change in surface viscosity is obtained with a 20 per cent. lipid concentration, which is similar to the lipid concentration occurring in natural membranes.

V. INTERACTIONS OF MONOLAYERS WITH SUBSTANCES INJECTED INTO THE UNDERLYING SOLUTION AND THEIR BIOLOGICAL ANALOGIES.⁷

a. Penetration of Lipoid and Protein Monolayers.

Research in this field consisted mainly in forming monolayers at interfaces composed of substances which exist in natural membranes and injecting into the underlying solution substances which are known to react with these membranes. A proof of the analogy with biological

systems was achieved by an identity of the specificity of the synthetic and biological reactions.

If certain soluble capillary active substances are injected in minute concentrations (10^{-4} per cent.) under a monolayer kept at a constant area and there is association by short-range forces between the film-forming molecules and the injected molecules, great changes in surface pressure, surface potential, and viscosity of the monolayer are observed. Furthermore, if the monolayer is kept at a constant pressure, great changes in the area of the monolayer are observed to take place.

These changes are shown to take place when the injected substance does not in itself affect the free surface of the aqueous solution to any great extent.

It has been shown that this reaction is very specific, and is due primarily to an interaction between the polar groups of the film-forming molecule and the injected molecule and, further, to association between the non-polar portions of the two molecules.

The polar group interaction anchors a molecule from the solution, thus permitting the non-polar portion to penetrate into the monolayer if there is association between the non-polar portions of the molecules. On penetration of a monolayer, therefore, the number of molecules is increased, and since the stability of the complex is greater than that of either of the two components alone, an equimolecular mixed film will result.

This explains why on penetration the surface pressure rises to very high values since the number of molecules in the monolayer has been doubled and the area kept constant. Further, the surface potential will rise or fall according to the magnitude of the penetrating polar group and its association with the polar group of the film-forming molecule.

b. Polar Group Specificity.

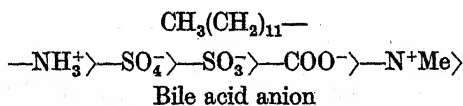
Excellent examples showing the specificity in reactions involving primarily the polar groups are given by the penetration of cholesterol and cholesterol ester monolayers by long chain hydrocarbon ions.

If a cholesterol monolayer be compressed to 2 dynes/cm. and a minute quantity of sodium hexadecyl sulphate be injected into the underlying solution, the surface pressure of the cholesterol monolayer rises within 2 minutes to *ca.* 55 dynes/cm., but if a film of cholesterol stearate or acetate be taken instead, the surface pressure rises only slightly above that of the sodium hexadecyl sulphate solution alone in the same time.

This penetration of the cholesterol monolayer was attributed to a complex resulting between cholesterol and sodium hexadecyl sulphate since a solution of saponin under identical conditions also raises the surface pressure of the cholesterol film to 60 dynes/cm. and solidifies

the film. This reaction is completely stopped with a film of cholesterol acetate. The saponin complex with cholesterol is well known, and it is interesting that saponin has been shown not to penetrate any other film such as lecithin, long chain alcohols, calciferol long chain phenols, sphingomyelin, &c., with which it is known not to make complexes. It is further shown that if equimolecular solutions of long chain paraffin ionic compounds of identical chain length be injected into a solution under a cholesterol film held at a constant area, then a reactivity series could be established with the different polar groups as measured by the equilibrium surface pressure of the cholesterol film.

This series is the following :



The long chain C_{12} amine salt penetrates a cholesterol film to the extent of 60 dynes/cm. and the bile acid salt has very little effect on the cholesterol film at all. The significance of these reactions will be demonstrated in the examples quoted later for their cytolytic action.

It appears that the attractive and repulsive forces between polar groups may be interpreted upon the hypothesis that they are due to coulomb forces acting between polar groups in systems containing ions and dipoles, an $\text{ion}^+ \text{-ion}^-$ having a greater energy of association than an ion-dipole, which again is greater than a dipole-dipole, and repulsion taking place between ions of like sign. It is these forces which are responsible together with the non-polar associations for the physical state of mixed films. Mixed films can be partially ionized acids or bases or two component systems such as acids and bases or mixtures of these with alcohols, ethers, esters, &c.

c. Non-polar Specificity.

The energy of the non-polar association depends on the number of (CH_2) groups or equivalent groups one can orientate and adlineate between molecules in a monolayer. Thus if 16 (CH_2) groups can be orientated opposite one another in molecules in a monolayer as in a crystal, the energy of association is equivalent to strong polar group interaction. The energy of association is dependent on the eighth power of the distance separating the carbon atoms, hence change in the adlineation due to stereochemical configurations would have a very great effect on the association between the molecules. These factors are well demonstrated in practice.

The penetration of a monolayer of cholesterol or long chain paraffin alcohol of sixteen carbon atoms by the salt of a long chain sulphate is very different according to the number of carbons in the penetrating

molecule. Thus it only requires a surface pressure of 8 dynes/cm. to eject C_{18} long chain sulphate molecules from a mixed film containing cholesterol, but already 35 dynes/cm. is required to eject C_{18} long chain sulphate molecules. Similarly great differences are observed between long chain compounds containing a double bond as to whether the arrangement is in the cis or trans configuration. The molecules of the cis compounds cannot adlineate either with themselves or other molecules in the trans configuration. Therefore on compression of a mixed film of a cis and trans compound ejection of the molecules of the cis compound will take place if the polar association between the molecules is weak.

This is again reflected in penetration experiments. Only very poor penetration of a monolayer of molecules in the trans configuration by molecules in the cis configuration can take place and likewise for the inverse order.

No ejection of a component takes place in a mixed film where strong association exists. Such films collapse as a 1:1 unit at very high surface pressures.

d. Penetration of Protein Films.

Long chain paraffin ionic salts of the description mentioned in this section have the property when injected into solutions under protein films of liquefying the protein film and ultimately dispersing it.

This occurs in very small concentrations; and the reactivity is similar but not so marked as the series quoted for penetration into cholesterol films.

The explanation given for this phenomenon is that the large protein molecules are associating in the form of a network through polar groups of the $-NH_3^+$ and COO^- type and also by their non-polar side chains. These associations will be broken down by competition from the polar groups of the penetration molecules. These smaller units will be deterged or displaced from the surface by the injected solution of the long chain paraffin salt which usually lowers the interfacial tension to a greater extent than the protein film. The bile acid salts seem to have no effect on protein films as with the sterol films, only associating with fatty acids.

e. Adsorption on to Protein Monolayers.

Penetration has been shown to change the surface pressure of the film and the surface potential. Adsorption, being primarily an association of the polar groups of the film-forming material and the injected substance, is shown only by a change in the surface potential and the viscosity or rigidity of the film.

The interest of this technique is the adsorption of material on to protein films. It is shown that the chief reacting group in the protein monolayer is the free NH_3^+ group, since most reactions undergone by protein monolayers are very similar to long chain amine films. The carboxyl and keto imido polar groups play a secondary role in the reactivity of the protein films. One of the most interesting reactions studied is the action of compounds with phenolic groups such as tannic acid and *p-p'* dihydroxy stilbenes, &c., on to protein monolayers. The reaction is related to the number of phenolic groups and their spacing per molecule of adsorbing compound.

Thus gallic acid adsorbs very slowly on to a protein monolayer, but tannic acid in very dilute concentration immediately reacts, changing the surface potential of a gliadin film from 360 to 200 mV. This tanned monolayer loses all its elasticity and behaves as a skin on the surface. It is also interesting that such a tanned monolayer can no longer be dispersed by long chain paraffin ionic compounds such as sodium oleate as explained in the previous section. But the gallic acid treated protein film can be dispersed in the usual way. This tanning reaction is very sensitive to pH. Optimum tanning taking place between pH 7-8, very little tanning takes place beyond pH 4 and 9.5.

It is supposed that the multiple-point contact of a tannic acid molecule interlaces the protein film by associating with the ionized amine polar groups in the film, thus preventing dispersion of the film and giving it extra rigidity.

The rate of the reaction is dependent on the number of reacting groups per adsorbing molecule. A long chain amine film is tanned in a similar way, but with a great expansion of the amine film; this does not take place with the protein films.

Pyrogallol is not adsorbed by protein films, but its polymerized product purpuro gallin is strongly adsorbed.

The *p-p'* dihydroxy stilbene and related compounds, which are synthetic oestrones, behave in an interesting manner, since with addition of side chains the adsorption increases markedly per added CH_2 group. The diethyl gives the maximum adsorption, since any further addition of CH_2 groups changes the adsorbing molecule into a protein film dispersant. It is interesting that the biological activity is at a peak with the maximum adsorbing compound. The dispersing properties of the compound coincide with the marked fall in biological activity.

f. Cytolysis and Agglutination.

It was quickly seen in seeking for biological analogies between the monolayer reactions and biological reactions that reactions taking place at cell surfaces were good examples, such as the cytolysis of red cells and unicellular animals.

All compounds examined that penetrate cholesterol monolayers or disperse protein films haemolyse, and all compounds that only adsorbed on to protein films without dispersing them agglutinate red cells.

Furthermore, the same reactivity series for long chain hydrocarbon compounds in penetrating cholesterol monolayers is very similar to the reactivity series obtained with these compounds in their haemolytic activity as measured by the minimum concentration necessary to produce haemolysis.

Thus long chain amines showed maximum haemolysis and the bile acid salts were not haemolytic at all.

Some of the unicellular animals such as *Paramecia* showed likewise a very similar behaviour to the red cells; instead of lysis, lethal activity as measured by movement of the animals was considered.

They were killed most readily by the long chain amines and least by the bile acids, and showed distinct cytolysis when dead.

Those substances which adsorbed on to protein monolayers without dispersing them agglutinated the red cells and killed the *Paramecia* without cytolysis.

These substances are the phenolic compounds as described, gallic acid having little action, but tannic acid is active in low concentrations. Likewise the *p-p'* dihydroxy-stilbene derivatives gave very similar values as to their adsorption on to protein films and their lethal action on *Paramecia*. The compounds as described which showed protein film dispersion properties were haemolytic and cytolysed the *Paramecia* in very low concentrations.

Rotifera, another microscopic animal, seemed to be immune against the substances mentioned here.

In view of these results it could be supposed that, since substances which penetrate cholesterol films and protein films are haemolytic and the substances which only adsorb on to protein films agglutinate red cells, there must be cholesterol and protein available on the red cell surface. It could thus be imagined, therefore, that the cell surface was a mixed cholesterol protein film. A similar surface would be feasible on these grounds for the *Paramecia* coating. The *Rotifera* which appeared to resist these substances must have another surface, such as chitin, which would be unimpaired by the lytic or adsorbing substances described here.

vi. THE OIL/WATER INTERFACE²

a. Emulsions.

Measurements on films and reactions these films may undergo with molecules in the oil or water phase can be made with similar techniques to those already described and also with the ring technique. The ring technique consists in measuring the force necessary to pull a platinum

wire through the interface. This is done by a torsion wire or a chainomatic balance.

Another and very useful method is by the ease of emulsification of the oil in water. The spontaneous formation, stability, type, and viscosity of emulsions depend on the following factors, which are all related to the interfacial film.

A very low interfacial tension is necessary to cause spontaneous emulsion. This is obtained as at the air/water interface by the formation of complexes. In this case the cholesterol or other oil-soluble component dissolved in the oil and the water-soluble component such as the sodium hexadecyl sulphate meet upon shaking the system and form a mixed film of great stability at the interface. The interfacial tension of these systems is less than 0.1 dyne.

If the interfacial film is fluid, as in this case, the viscosity of the emulsion is low, but if a long chain alcohol such as hexadecyl alcohol is substituted for the cholesterol, the adlineation between the molecules permits the interfacial film to go solid. This makes the emulsion into a thick grease.

b. Type of Emulsion

If the interfacial film is charged, permitting the oil droplet to possess a diffuse ionized layer, then the emulsion will be of the oil in water type, since this will result in repulsion between charged oil droplets. Removal of this charge breaks the emulsion, but if at the same time a rigid or highly viscous uncharged interfacial film is formed, then a water in oil emulsion is made.

A typical example of this is to stabilize an oil in water emulsion by dissolving cholesterol into the oil phase and crowding to the interface long chain amine ions dissolved into the aqueous phase by complex formation: this stabilizes an oil in water emulsion. It is simple to invert this emulsion to a water in oil type by addition of tannic acid in neutral solution. Tannic acid neutralizes the charge of the amine and solidifies the interfacial film.

It can be shown with the emulsion technique that all the reactions taking place at the air/water interface also take place at the oil/water interface and confirm the same degree of specificity in the formation of complexes at the interface.

c. Adsorption of Proteins at the Oil/Water Interface.

Proteins spread more readily at the oil/water than at the air/water interface, and can be shown to have quite different properties when adsorbed, other than those described for the air/water interface.

It has been very recently shown by A. C. Frazer (London)² that

proteins such as insulin, and bacterial and other toxins, when adsorbed at the oil interface, reversibly lose their biological activity.

Thus olive-oil emulsions stabilized by the proteins cause the protein to show none of its biological action, but when the emulsion is broken the reactivity returns. This is an excellent example where orientation of the protein molecule in a film governs the reactivity of the protein.

It appears also that globulins are more readily and preferentially adsorbed at the oil interface than the albumens.

Another example where orientation of the molecules at the interface controls the rate of reaction is with hydrolysis of aliphatic esters, both by OH' ions and ferments.

At the beginning of this chapter two configurations are given for an ester, first, where the two hydrocarbon chains are adlineated together in a hairpin form: in this configuration hydrolysis of the esters by caustic soda or by pancreatin can take place; secondly, where the hydrocarbon chains are opposed to one another: here very restricted hydrolysis of the ester takes place even when the one chain is composed of only two (CH_2) groups. The orientation of the molecule can be made in a monolayer by compression of the film and with an emulsion by suitable choice of the ester; thus ethyl benzoate is undigested but benzyl propionate is readily digested by pancreatin.

CHAPTER III

THE CELL SURFACE AND CELL PHYSIOLOGY

By J. F. DANIELLI

i. INTRODUCTION

IT is not intended here to cover all the ramifications of this subject, but rather to deal particularly with those branches in which there have been substantial advances in the last twenty years (i.e. since Bayliss wrote the last edition of his *General Physiology*)² and also with certain other branches in which many of the preconditions for such an advance appear to exist. During the course of this chapter it will become clear, if it is not already so, that there is a hiatus between the cell surface and the bulk of the cytoplasm and that the relationships between events occurring in these two parts of the cell are but little explored. In this respect a difficulty and gap in our knowledge occurs, analogous to the situation in nerve physiology some years back, when it was known that an impulse in one nerve axon, on reaching a synapse elicited a response in a second axon, but the mechanism of transmission across the synapse was unknown.

When considering the cell surface it is the region reaching inwards from the plasma membrane with which we are concerned. Many cells have supporting or protecting membranes outside the plasma membrane, but micro-dissection has shown that these outer membranes are not essential for life, whereas the plasma membrane cannot be removed, or even badly damaged, without death of the cell. We must therefore regard the plasma membrane as a part of the cell as essential as, for example, the nucleus. Reaching inwards from the plasma membrane is a more or less narrow band, the ectoplasm, which is usually gelled and relatively free from cytoplasmic granules, &c. (Fig. 1). In many ways this layer is closely similar to the bulk of the cytoplasm, but it is also directly concerned in, for example, cell division, and so comes into the picture of the cell surface also.

ii. STRUCTURE AND PHYSICAL PROPERTIES OF THE PLASMA MEMBRANE

a. *Molecular Structure.*

The great majority of cells studied have a common ground-plan for the structure of their plasma membranes which we are now able to describe, though our description is crude and includes no detail and no consideration of local differentiation of the membrane. In this group we can include with certainty such cells as erythrocytes, nerve axons,

muscle fibres, leucocytes, yeast, *Nitella*, many cells of higher plants, and marine ova such as those of sea-urchins and starfish. On the other hand, permeability measurements suggest that the sulphur bacterium *Beggiatoa mirabilis* has a membrane of rather different structure. Virus particles differ from cells in having no plasma membrane and are there-

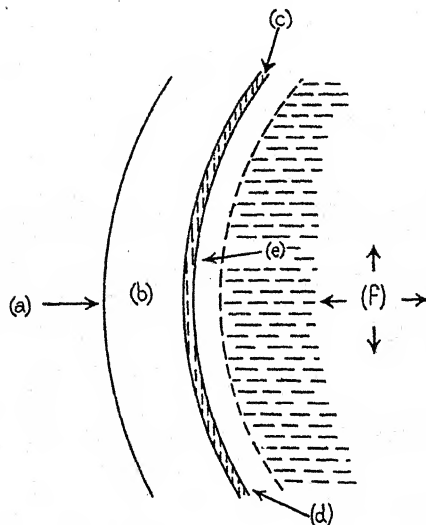


FIG. 1. Diagram of surface layers of a fertilized *Arbacia* egg.

a. Fertilization membrane. b. Perivitelline space. c. Hyaline plasma layer. d. Plasma membrane. e. Gelled cortex or ectoplasm. f. Endoplasm.

fore unable to control their internal composition in the same way that cells do.

The three fundamental properties of the membranes of the main group of cells, from which we proceed to define the membrane structure, are (1) preferential permeability to lipid-soluble substances, (2) the existence of a low tension at the surface, (3) high electrical resistance. Properties (1) and (3) indicate that there must be a continuous layer in the membrane composed of lipid molecules, such as phosphatides, sterols, and fats. Property (2) indicates that at the surfaces of the membrane there is an adsorbed protein layer. The arrangement shown in Fig. 2 is the simplest possible structure which can meet these essential requirements. It consists of a continuous film of lipid molecules, of which the two outermost layers are so orientated that the hydrated polar groups are in the oil/water interfaces, with a layer of protein molecules adsorbed on both of these interfaces. It cannot yet be said to what extent the protein is mechanically superimposed upon the lipid, and

to what extent the surface must be regarded as a specific protein-lipoid complex.* With this arrangement of molecules the tendency of the lipoid molecules to change their orientation will be insignificant and since the membrane is a continuous surface (a spherical shell) all surface-tension forces will balance out and will not tend to disrupt the film.

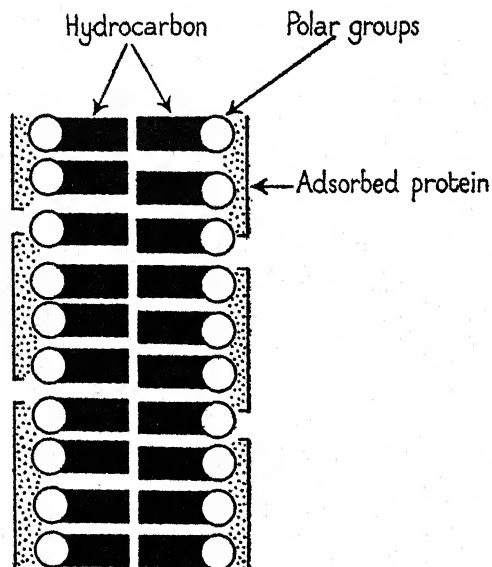


FIG. 2. Diagram of molecular structure of the plasma membrane.

Spherical shell membranes of this type can be made experimentally and show considerable stability.

Various other arrangements of the lipoid molecules in a continuous lipid film are conceivable, all of which, however, are less satisfactory, since they both tend to revert to the type of Fig. 2, and also to coagulate irreversibly the proteins adsorbed upon them. These cases are discussed in detail elsewhere (Danielli, 1936^{16a}).

The adsorbed layers of protein shown in Fig. 2 are the primary adsorbed layers, consisting of denatured (possibly reversibly denatured) protein. The protein in these layers consists of polypeptide chains, or meshworks of such chains, lying in the plane of the interface, with the hydrocarbon portions of the amino-acid residues dissolved in the lipid layer and the polar groups in the aqueous phase. There may be a further layer of globular protein adsorbed on to this primary layer; it is almost certain that this will be so in all cases on the inner (cytoplasmic) surface of the membrane. Since the adsorption of globular proteins

* See Chapter II.

is readily reversible, the presence of globular proteins on the outer surface will depend on the nature of the bathing fluid; when this is plasma, cells usually behave as though coated with globulin.

The mechanical properties of the adsorbed polypeptide chains are probably largely responsible for the elasticity and relatively great mechanical strength of the plasma membrane. A single polypeptide chain may be 50 m μ or more in length and will be attached to similar chains by hydrogen bonds and other linkages and will thus 'tie' the various parts of the membrane together, but, owing to the elastic properties conferred by such interlinked chains, without conferring upon the membrane an undesired brittleness.

That the cell surface is a lipid layer was first suggested by Overton, as a result of his work on cell permeability, but difficulties in the interpretation of permeability studies soon led to a suggestion from Nathanson that the cell membrane is really a mosaic of areas of different properties, some adapted to permit passage of one type of molecules, others to permit passage of quite different molecular types. This suggestion has fluctuated in popularity, but evidence is accumulating to show that, whilst the continuous lipid layer described above is the basic pattern of the cell membrane, there are relatively small areas which are specially differentiated, so that we are beginning to adopt Nathanson's suggestion in a somewhat modified form. These special areas or patches in some cases at least are comparable with the active patches of a catalyst: for example, Jacobs and his colleagues have found that certain erythrocytes are abnormally permeable to glycerol, but that acidity, or a minute trace of copper, will reduce the permeability to a value typical of most other cells. From the kinetics of penetration it also follows (Danielli³¹) that the areas permitting abnormal permeation are a small fraction only of the total membrane area. One of the chief tasks before workers on cell permeability is to discover the extent, structure, and function of these specialized patches.

A further counter-suggestion to Overton's hypothesis was that the cell membrane is a molecular sieve, containing water-filled pores, through which molecules of diameter less than the pore diameter would diffuse quite readily. For most cells this theory is now discredited, the only exception being that of *Beggiatoa*, for which diffusion kinetics suggest that the molecular sieve hypothesis is correct. This, however, is still by no means certain.³¹

b. Thickness.

The thickness of the plasma membrane has been calculated by four methods. Gorter and Grendel⁴⁰ have found that the total lipid extractable by lipid solvents of the erythrocytes of various species would make a layer not more than bimolecular in thickness. Consequently, in

the case of the erythrocyte there is a clearly defined upper limit to the thickness (but not a lower limit). It is possible that Gorter's choice of solvents has led to a low value, but not more than 50 per cent. low.

The second and third lines of approach are from the impedance of cell suspensions. Fricke³⁶ has shown, from the capacity of the cell membrane, that the thickness of the erythrocyte wall is of the order of 50 Å; Fricke and Curtis have obtained a similar value for polymorphonuclear leucocytes³⁸ and for yeast cells.³⁷ Cole's results^{11,12} with the eggs of *Arbacia*, *Asterias*, and *Hipponoe* also give values of the same order. However, as these authors have pointed out, the analysis leading to this conclusion is far from unequivocal. The agreement between Fricke and Gorter and Grendel has been considered to place a satisfactory upper limit of 2-4 molecules to the thickness of the lipid layer in erythrocytes. It is improbable that the values obtained for the other cells are in error by a factor of more than tenfold.

From the resistance of the cell membrane it can be calculated that the thickness lies between 1 μ and 1 $m\mu$.

The fourth line of approach is that of Schmidt *et al.*^{87,88}, who conclude that the red cell envelope consists of a few layers of radially orientated lipid molecules and a number of tangentially orientated protein polypeptide chains. These results were obtained from birefringence studies.

From these investigations it appears improbable that any cell yet studied has a lipid layer thicker than 10 $m\mu$.

c. Ionic Character.

The fatty molecules adsorbed at the two oil/water interfaces of the membrane are oriented so that their polar groups will be directed, on the average, with the positive end of their electrostatic dipoles towards the oil layer and with the negative end towards the aqueous phase. Owing to this orientation of electrical dipoles, ions from the surrounding aqueous phase will distribute themselves, negative ions inside the oil layer around the positive ends of the dipoles, positive ions in the aqueous phase around the negative ends of the dipoles. Thus there will be an excess of negative ions inside the oil layer (Davson and Danielli²⁹). The nature of these ions may exert an influence on the structure and behaviour of the membrane, though the evidence available suggests that this influence is small compared with that of the cations in the aqueous phase.

In the aqueous phase will be certain cations oriented round the negative ends of the dipoles of the polar molecules, and also other cations distributed around the carboxyl* and phosphatide* ions in the surface. Thus, in the aqueous phase in the immediate vicinity of the cell membrane there will be (a) an excess of mobile positive ions over mobile

* Attached to the lipins and proteins.

anions and (b) an excess of cations over the concentration in the bulk aqueous phase bathing the cell (Fig. 3). The thickness of this region of excess ions may be calculated from the theory of Debye and Hückel³³ (1923). At 20° C. in solutions of uni-univalent salts the thickness is

$$\delta = \frac{3.1 \times 10^{-8}}{\sqrt{C}} \text{ cm.}$$

For $C = 0.1$ molar, $\delta = 0.98 \text{ m}\mu$, and for $C = 0.0001$, $\delta = 31 \text{ m}\mu$. Thus in dilute solution this region of excess ions may be as thick or

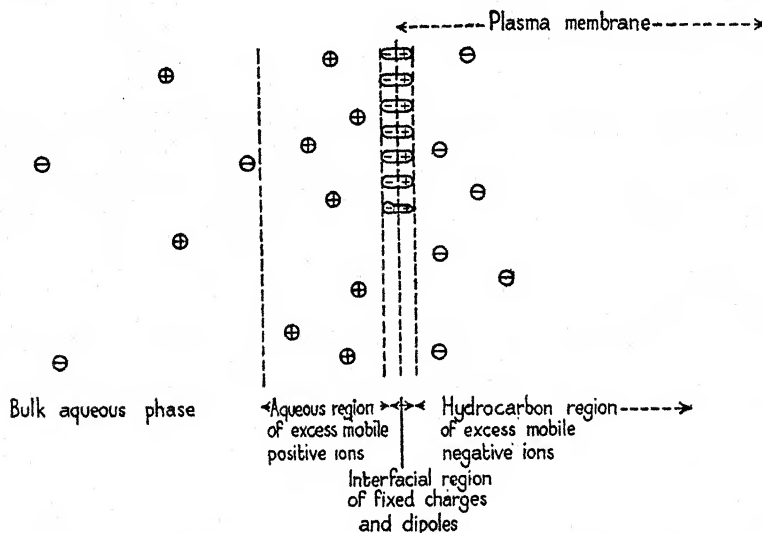


FIG. 3. Diagram of electrical structure of the plasma membrane.

thicker than the lipid layer itself. The concentration of the various ions at the surface of the cell may be calculated from the Gibbs-Donnan equilibrium (Danielli^{17,24}) or from the electrokinetic potential (Hartley and Roe⁴⁴). At the surfaces of most cells the ratio, average concentration of univalent cations in surface layer : concentration of cations in normal environment, lies between 1.1 and 5. Since this applies to hydrogen ions as well as K^+ , Na^+ , &c., the pH at the surface of a cell is usually between 0.1 and 0.7 pH units different from that of the medium. The inverse rule applies to mobile anions; there will be a deficiency of mobile anions in the aqueous layer adjacent to the membrane, so that the ratio, average concentration of univalent anions in surface : average concentrations of anions in normal medium, lies between 1/1.1 and 1/5.

It is theoretically possible that locally the excess of cations in the surface may exceed the values indicated by the ratios just given. In artificial systems values as high as 200 have been found for this ratio (Webb and Danielli⁹⁷). Wilbrandt⁹⁸ has pointed out that when divalent

ions are considered the excess concentration in the surface becomes even more marked, for it follows from the Gibbs-Donnan equilibrium that the relationship between the univalent and divalent ion concentrations is that

$$\text{if } \frac{[\text{Na}]_{\text{surface}}}{[\text{Na}]_{\text{bulk}}} = r,$$

$$\text{then } \frac{[\text{Ca}]_{\text{surface}}}{[\text{Ca}]_{\text{bulk}}} = r^2.$$

Hence the ratios for calcium concentrations for cells in their normal environment lie between $1 \cdot 1^2$ and 5^2 , or i.e. between 1.2 and 25, according to the cell. Webb and Danielli⁹⁷ showed that the surface excess of total calcium in the surface may exceed the values calculated in this way, for the calculation just given refers to *ionic* calcium only, and in addition calcium may be held in a surface in an apparently non-ionic form. Owing to the operation of these two factors it is found that at the surface of most cells, when the external medium contains ions giving a Na:Ca ratio of about 100:1, the calculated ratio in the surface may be roughly 1:1 (Danielli²³). This may be a very important fact, for the work of Ringer, Loeb and many others has shown that for a cell to behave normally it must be bathed by a solution containing a Na:Ca ratio of the order of 100. Usually a type of cellular behaviour dependent on the cell surface is at one extreme in solutions containing NaCl only, at another extreme in CaCl_2 , and a balance between the two is achieved when Na:Ca = 100. It now seems possible that the reason for this value for the bulk ratio is that this bulk ratio is what is necessary to give a ratio of *c.* 1 in the surface, where the balancing action has to occur. It is quite impossible for one Ca ion to counterbalance 100 Na ions, but when the ratio is near to unity this difficulty vanishes. Some caution must be observed in using this theory, for at present the evidence that the surface ratio is close to unity is indirect, based on calculations and not direct experimental evidence.

d. Lysis.

There are innumerable ways in which lysis may be brought about and many lytic agents cannot penetrate the cell membrane, so that, with such agents, lysis must be the result of direct action upon the cell membrane. In these cases, as Davson²⁸ suggests, we are carrying out a partially controlled degradation of the cell membrane, somewhat analogous to degradative analysis in organic chemistry. The most significant types of lysis, from our present point of view, are produced by (a) antibodies, (b) polyhydroxylic phenols, (c) heavy metals, (d) lipid solvents, (e) lecithinase, (f) digitonin, (g) sodium or potassium salts of fatty acids and paraffin sulphonates ($\text{C}_n\text{H}_{2n+1}\text{SO}_3\text{Na}$). The first two of

these reagents probably act almost exclusively on the protein part of the membrane and the latter four mainly on the lipid part of the membrane. Heavy metals probably act on both lipoids and on proteins.

Antisera to cells will contain antibodies to the adsorbed protein on the cell surface, and it is presumably to these that the lytic action is due, as it is very doubtful whether antisera to lipoids can be made. The antibodies, by combining with the membrane proteins, will break down other complexes existing between the protein and the membrane lipid molecules, and so produce a general disruption of structure. The polymerized polyhydroxylic phenols exert a tanning action on protein films (Schulman and Rideal⁵⁹) and consequently will also disrupt the structure in much the same way. The simple polyhydroxylic phenols may act in a similar way (Davson and Danielli³⁰), but it is difficult in such cases to be sure that the lytic action is not due to the slow formation of polymers. The lytic action of these two types of substances which attack protein primarily shows the great importance of the primary adsorbed protein layer.

Heavy metals probably act by combining with the carboxyl and phosphate groups of the proteins and lipoids, to give non-ionic compounds, and probably also causing closer packing of certain types of molecules than is normal. It is most interesting in this connexion that Jones⁵⁹ has found that the toxic action of the heavy metals is proportional to their solution pressure, i.e. the more readily a metal forms unionized complexes with anions, the greater is the toxic action of the metal.

Of the remaining lytic agents, lipid solvents probably act by dissolving in the lipid layer and breaking down its structure by solvent action; lecithinase by converting lecithin into lysolecithin (which is itself lytic); digitonin by forming a complex with the membrane sterols; and the salts of fatty acids and paraffin sulphonic acids by forming complexes with the sterols, &c., though it is possible that they also react with the protein layer. Schulman, Rideal and their colleagues have shown that reactions of the last three types will all occur in monolayers.

Thus the action of these reagents for lipoids shows that the lipid part of the membrane must also retain its normal structure within comparatively narrow limits, if the membrane is to remain stable.

e. Surface Tension and Elastic Properties.

The surface tension of cells, more properly called the tension at the surface, has frequently been invoked to explain cell division. The tension at the surface is defined as the sum of the elastic tensions and surface tensions of the plasma membrane. There are two ways in which surface tension may affect cell division. (a) A belt of the surface in the

cleavage plane of a cell may have its surface tension raised; the result would be that the inwardly directed pressure in the vicinity of the cleavage plane would exceed that elsewhere, so that cleavage would occur if the rise in tension were sufficient. But it is very difficult to believe that this can occur. In the first place, during cleavage the cortical gel layer in many species has marked rigidity, so that a very considerable rise in tension would be required to cut through the cortex. In the second place, local differences of tension in an area less than 1 sq. cm. vanish in a fraction of a second, whereas cleavage may take many minutes, so that for division to result from local tension changes it would be necessary to provide for continuous localized production of a substance capable of raising the tension at a lipo-protein surface by many dynes; there are many reasons for regarding this as improbable. Thirdly the cell contains, and is usually bathed by fluids containing, many substances of great surface activity which should prevent such rises in tension occurring. (b) Alternatively, division may occur if the cell were elongated, by, for example, growth of the spindle, to a length π (3.14) times the diameter of the cell, since such a cylinder divides spontaneously, without any local changes in tension being necessary. But Table I shows that in fact cells divide long before they have elongated to this extent, so that this alternative can be immediately discarded.

TABLE I. *The ratio of the long axis (l) to the short axis (b) at the time of initiation of the cleavage furrow in various cells. Spontaneous division would occur if $l/b \geq \pi$*

Cell	l/b	Authority
Egg of <i>Echinus esculentus</i> in sea-water	1.5	Gray (1924) ⁴²
Ditto in Ca-free sea-water	1.9	Gray (1924) ⁴²
Egg of <i>Arbacia punctulata</i>	1.4	Just (1939) ⁶⁰
Cell from choroid of chick's embryo	2.0	Strangeways (1922) ⁸⁴
Spermatocytes of <i>Hemiptera</i>	1.35	Bowen (1920) ⁴

Further light has been thrown on this problem by direct measurements of the tension at the surface of cells. Early attempts at such measurements were invalidated by experimental errors, and the successful techniques are mainly due to E. N. Harvey and to K. S. Cole and their colleagues. Many techniques have been used,* of which the centrifuge method, the compression method and the kinetic method are typical. The centrifuge method may, for example, be applied to marine eggs in which, under centrifugal force, a stratification of the cell contents occurs, the lighter parts moving to the centripetal end of the egg and the heavier parts to the centrifugal end. When the separation has occurred the difference in density between the two ends of the egg gives

* See review, Harvey and Danielli⁴⁷.

rise in a centrifugal field to forces elongating the egg into a cylinder, which divides into halves when its length exceeds π times its diameter. Then, knowing the minimum centrifugal force necessary to obtain sufficient elongation and the density difference between the two halves, we can calculate the force required to elongate the egg, and equate this to the tension round the circumference of the elongated cylinder. For the unfertilized *Arbacia* egg the tension at the surface found by this method is 0.2 dynes/cm., an extremely low value (Harvey⁴⁵).

Cole¹⁰ compressed *Arbacia* eggs with a minute gold beam. From the pressure exerted by the beam he was able to calculate the internal pressure of the egg (due to the surface tension) and from this to calculate the surface tension. He obtained a value of 0.08 dynes/cm. for the uncompressed egg by extrapolation of the tension-compression curve to zero pressure. The membrane was definitely elastic. Harvey and Danielli⁴⁶ studied some of the types of surface systems which might occur in the plasma membrane, finding that all surfaces containing protein, and only surfaces containing protein, have elastic properties. Cole's demonstration of elasticity in the plasma membrane may therefore be regarded as supporting the view that protein is adsorbed on the cell surface.

Sichel and Burton⁹² showed that the tension at the surface of the blastomeres formed by the first cleavage of *Arbacia* eggs has a similar value, 0.09 dynes/cm. They followed the cleavage of the egg until the blastomeres were connected by a small stalk only, then punctured one of the blastomeres and took moving pictures of the decrease in volume of the other blastomere as its contents discharged through the stalk. From the rate of discharge, assuming Poiseuille's law held, the excess pressure inside the egg, due to surface tension, was calculated; from this pressure the surface tension was derived. Evidence was also found of elastic properties of the cell surface which may, however, in these measurements be due to other causes.

Another method, from Harvey's laboratory, is to stretch a cell between two micro-needles and calculate the tension from the force necessary to obtain a given degree of elongation of the cell. Norris⁹¹ obtained values of the tension of the surface of c. 0.1 dynes/cm. for *Arbacia* eggs and of less than 0.1 dynes/cm. for erythrocytes.

Results for a number of different cells are shown in Table II. The tension at the surface is always lower than 2.0 dynes and often less than 0.2 dynes/cm. These low tensions seemed at first incompatible with the view that the cell membrane is a thin lipid layer, as natural lipoids have much higher tensions against sea-water than, for example, the 0.1 dynes per cm. found for *Arbacia* eggs (actually 0.05 dynes at each side of the membrane). However, Harvey and Shapiro⁵¹ showed that intracellular oil droplets in fish eggs also had very low surface tensions and had elastic surfaces—as has been more recently shown for oil

droplets in *Amoeba proteus* and in eggs of the fresh-water crustacean *Daphnia pulex* (Harvey and Schoepfle⁵⁰)—and Danielli and Harvey²⁶ showed that the substances responsible are proteins, which by adsorption on the lipid surfaces both reduce the tension at the surface to extremely low values and also render the surface elastic.

It will be seen that these studies of the tension at the cell surface have been most useful in developing our knowledge of the structure of the cell membrane.

TABLE II. *The tension at the surface of various cells*

Cell	Tension dynes/cm.	Authority
<i>Arbacia punctulata</i> : egg . . .	0.2	Harvey, 1932 ⁴⁵
	0.08	Cole, 1932 ¹⁰
	0.09	Sichel and Burton, 1936 ⁵²
	0.1	Norris, 1939 ⁸¹
<i>Triturus pyrrhogaster</i> : erythrocyte	0.1	Norris, 1939 ⁸¹
<i>Amoeba dubia</i>	1-3	Harvey and Marsland, 1932 ⁴⁹
<i>Physarum polycephalum</i>	0.45	Vexler, 1935 ⁹⁸
Rabbit macrophage	2.0	Shapiro and Harvey, 1936 ⁸¹
Frog leucocytes	1.3	Shapiro & Harvey, 1936 ⁸¹
<i>Busicon caniculatum</i> : egg . . .	0.5	Harvey & Fankhauser, 1933 ⁴⁸
<i>Triturus viridescens</i> : egg . . .	0.1	Harvey and Fankhauser 1933 ⁴⁸

f. Wetting Properties.

Some very interesting results have been obtained by endeavouring to cause oil to coalesce with the cell membrane. Mudd and Mudd^{77,78} investigated the ease with which various types of cell are wetted by, and coalesce with, a film of oil advancing between a cover-slip and slide. Erythrocytes were readily wetted by the oil, but leucocytes have a hydrophilic surface and when engulfed by an advancing film of oil retain a thin layer of saline between the cell membrane and the oil. An oil droplet in saline is readily wetted by such a film of oil, but if protein is added to the saline the surface of the droplet becomes hydrophilic and such a droplet behaves like a leucocyte. Consequently Mudd and Mudd suggested that leucocytes are coated with protein, whereas erythrocytes have a surface layer of lipid material. From what we now know of proteins at the oil-water interface it can be said that the surfaces of both cells are probably coated with protein, but that, while the outermost layer at the surface of leucocytes is a strong elastic gel layer, probably serum globulin, the layer at the surface of erythrocytes is probably a weakly elastic or liquid layer, possibly of serum albumin, which is known to form comparatively fragile films. In this connexion it is interesting to find that Ponder and Furchgott⁸⁵ have found that maintenance of the discoid form of the mammalian erythrocyte is connected

with the adsorption of a crystallizable fraction of the serum albumin upon the erythrocyte. Reversible disk-sphere changes occur, according to whether this albumen is, or is not, present in the fluid bathing the erythrocyte.

Dawson and Belkin³² found that a globule of oil readily adheres to the surface of *Amoeba dubia*, forming a cap (Fig. 4). The cap is not drawn into the interior of the cell. Marsland⁶⁹ used this technique to

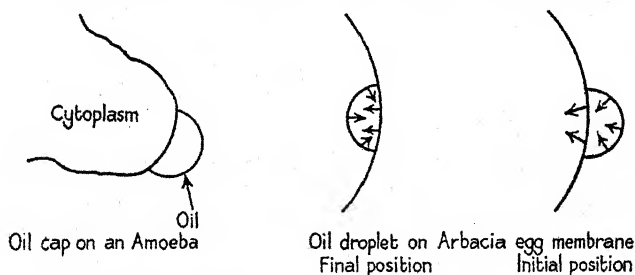


FIG. 4. Diagrams illustrating the behaviour of oil droplets brought into contact with the cell surface. The arrows indicate the internally directed force in the droplets due to interfacial tension.

investigate the site of narcosis by paraffin oils. The oils were diluted in olive oil and the solutions either injected into the interior of an *Amoeba* or fixed on its surface as a cap. At a sufficient dilution of paraffin with olive oil the injected droplets had no harmful effects on the *Amoebae*, but the caps still caused clearly defined narcosis. It seems beyond doubt that the site of narcosis in this instance is the cell membrane and that the paraffin dissolves in the lipid layer of the surface, as other lipid solvents do. Chambers⁸ and Kopac and Chambers⁶¹ have obtained equally convincing evidence with naked *Arbacia* eggs from which the pellicle has been removed. Here an applied droplet of a suitable oil 'snaps in' when applied to the surface, making a flattened surface of contact on the inside of the cell membrane, and *lifting the membrane off the granular cytoplasm*. By directing a stream of water from a micro-pipette against the cell surface, the membrane can be made to flow, and if two such 'snapped in' droplets are adhering to the membrane, they can be seen to move relatively to one another. It seems incontestable that the plasma membrane of *Arbacia* eggs is fatty or lipid in composition, and moreover is liquid.

The difference between *Amoeba dubia* and the egg of *Arbacia* seems to depend upon whether the ectoplasmic or cortical layer, underlying the plasma membrane, is a gel or not. If it is a rigid gel, capping occurs. If it is liquid or a weak gel, penetration occurs, owing to the surface tension outside the membrane being greater than that inside the cell. The surface tension is lower inside owing to the high concentration of proteins. With *Arbacia* penetration occurs, for example, only if

the interfacial tension of the penetrating oil is greater than about 9.5 dynes/cm. Thus, on touching the cell surface in protein-free sea-water the surface tension at the outer surface of the oil drop is about 9.5 dynes/cm., whereas adjacent to the ectoplasm it is about 0.05 dynes. Hence the internal pressure of the droplet due to the external interfacial tension will be, for a drop of 5μ in radius, about 4×10^4 dynes/cm.², and the interfacial pressure due to the side adjacent to the ectoplasm is only 2×10^2 dynes/cm.² This difference in pressure literally forces the drop through the plasma membrane. The critical tension of 9.5 dynes, giving a pressure of 4×10^4 dynes/cm., probably represents the pressure necessary to deform the cortical gel, lift the plasma membrane off the gel, and permit entry of the droplet. If the sea-water contains protein it adsorbs on the oil, the surface tension is reduced, and penetration cannot occur.

These experiments of Kopac and Chambers are particularly valuable, since they give that direct proof of the existence of a lipid layer at the cell surface in one case which many investigators have regarded as so essential. Many lines of indirect evidence have pointed to this conclusion for many different types of cell. This direct proof for the *Arbacia* egg enables us to utilize the indirect evidence available with other cells with much more confidence than would otherwise be the case.

g. Permeability.

In considering the permeability of the cell surface we must initially divide the field into two sections, the first dealing with simple diffusion which involves no expenditure of energy by the cell; the second dealing with assisted diffusion, or secretion, in which substances are redistributed wholly or partly as a result of cellular activity involving the utilization of energy. The first section can now be dealt with quantitatively, to a first approximation, but secretion is a field in which we are ignorant even of the outline of the processes involved.

The permeability of cells to non-electrolytes is a function of the character both of the cell membrane and of the penetrating molecule. The more important molecular characteristics involved are molecular weight and diameter and the nature, polar or non-polar, of the individual groups of the molecule. It is the balance between polar and non-polar groups which determines the oil/water partition coefficient of a molecule. Overton, in the last decade of the nineteenth century, found that permeability was a function of the oil/water partition coefficient of the penetrating molecules, so that molecules with a high oil/water partition coefficient are able to penetrate more rapidly than molecules with a small partition coefficient. This led Overton to suggest that the cell membrane is a thin lipid layer, and although many alternative hypotheses have been considered, for the great majority of cells for which we have experimental results this theory of Overton's is

undoubtedly correct. A possible exception is *Beggiatoa mirabilis*, the membrane of which may be a molecular sieve containing little lipid. But, as has been remarked above, special areas of certain cells are differentiated to permit penetration of certain particular molecules, so that the lipid layer is certainly not in all cases a complete homogeneous layer. Table III shows the permeability of the membranes of different cells to ethylene glycol, glycerol and erythritol, compared with the permeability of a roughly equal thickness ($5\text{ m}\mu$) of water. For such molecules the permeability of the cell membrane is at least 10^9 times less than the permeability of the same thickness of water. Very few physiologically important substances have a permeability greater than 10^{-8} of that of the water layer, significant exceptions being oxygen and carbon dioxide. It is most remarkable that a membrane only $5\text{ m}\mu$ in thickness should be able to bring about this reduction in diffusivity. It has been calculated (Danielli¹⁹) that a hundredfold increase in thickness would make comparatively little difference to the permeability of the cell membrane to many molecules, such as glycerol and glucose; the optimum efficiency is secured by a layer only a few molecules thick and further increase in thickness would be a comparatively expensive luxury.

Table III also shows that there is a considerable degree of specificity, different cells having different permeabilities to the same molecule.

Experimental results with ions show that here, also, there is considerable variation from cell to cell and even in the cases of greatest permeability to ions the cell membrane still reduces the permeability to a value not more than 10^{-8} of that of a water layer of the same thickness. Generally speaking, the smaller, i.e. the less hydrated, an ion the more readily it penetrates the cell membrane. Maizels⁶⁸ has shown that the permeability of erythrocytes to different anions is $\text{NO}_3 > \text{Cl} > \text{acetate} > \text{oxalate} > \text{sulphate} > \text{citrate}$: this is the order of increasing hydration. With regard to cations, it is known that with most cells the small ions potassium, rubidium, and caesium can penetrate much more rapidly than the large ions lithium and sodium. Davson²⁷ has shown that similar considerations come into play when simultaneous diffusion of both anions and cations is studied.* With regard to specificity: it is generally considered that erythrocytes are much more permeable to anions than to cations, and that most other cells, including muscle and nerve cells, are more permeable to potassium than to anions. In the view of the present writer, however, it is probable that most cells will be found to be comparatively readily permeable to small anions such as Cl^- , and that in addition many cells are also permeable to potassium and to a much less extent to sodium. From this point of view the 'specific'

* There is evidence that results in such cases are complicated by the phenomenon of 'anomalous' osmosis.

permeability of erythrocytes to anions merely reflects the fact that the erythrocyte is a moribund cell and has lost the attribute of permeability to potassium which it must have possessed in the reticulocyte stage, or in some earlier stage. The failure to observe anion permeability with, for example, muscle cells, in the case of ions such as Cl^- , is probably due to the equilibrium conditions determining the amount of Cl^- in the muscle cell being such as to practically exclude Cl^- from the interior of the cell. This view is supported by the fact that phosphate penetrates muscle fibres quite readily.

TABLE III. *Calculated permeability of a thin oil layer and experimental values of the permeability of some cells. Permeability in mols./sec./ μ^2 per mol. per litre concentration difference, $\times 10^{16}$*

	5m μ water, viscosity = 0.01 poise	5m μ oil, viscosity = 10 ³ poise	Ox erythro- cyte	Arbacia egg	Chara cerato- phylla	Plagio- thecium denticu- latum	Pylaiella litoralis
Ethylene glycol	1.7×10^3	0.1	0.2	0.73	1.2	0.03	0.1
Glycerol	1.4×10^3	0.002	0.002	0.005	0.02	0.0003	0.002
Erythritol	1.2×10^3	0.00005	0.001	0.00007	0.0001

When we turn to large molecules, such as proteins, sterols, and neutral fats, we find that in many cases penetration of the cell membrane does occur, but calculations show that penetration cannot occur by simple diffusion (Danielli²⁰). One can only conclude that such molecules enter the cell through specially differentiated areas, by special mechanisms, of which we are at present ignorant. A detailed review of the permeability of cells to ions and molecules is given by Davson and Danielli³¹.

The temperature coefficient of penetration of most molecules and ions is of the order of 2 or more per 10° C. rise in temperature. As diffusion in water has a Q_{10} of 1.2 to 1.4, these large Q_{10} values were at one time supposed to be due to transport across the cell membrane being dependent upon the mediation of a chemical reaction. We know now, however, that these large Q_{10} values may arise in simple diffusion processes; where diffusion is fast, the resistance to diffusion is small and the Q_{10} is small. Where diffusion is slow, the resistance to diffusion is large and the Q_{10} is large. These conclusions follow from a simple consideration of diffusion kinetics. The study of the kinetics of diffusion across a thin lipid layer has shown that in all qualitative and in many quantitative aspects simple diffusion is able to account for the rates of penetration of different molecules in most cases, and that only where penetration is shown to be associated with the utilization of energy by the cell need special chemical reactions be invoked. From these diffusion kinetics

studies it has been found possible to calculate the approximate permeability of a thin lipid layer. Some results of such calculations are shown in Table III for glycol, glycerol, and erythritol. The order of magnitude agrees well with that found for living cells.

The cell membrane presents three sites of resistance to free diffusion: (1) the membrane/water interface, for diffusion from water to membrane; (2) the membrane/water interface, for diffusion from the membrane into water; (3) the interior of the membrane. Every molecule penetrating into the interior of the cell has to pass through these three sites of resistance. A molecule with polar groups in it, e.g. hydroxyl (OH) groups, as opposed to, for example, non-polar groups such as methylene (CH_2), forms at least one hydrogen bond with water for each polar group. All these hydrogen bonds must be broken simultaneously if a molecule is to penetrate into the (lipid) membrane, since otherwise the molecule is not free to diffuse into the lipid layer. Thus glycerol, having three OH groups, must acquire sufficient kinetic energy to break three hydrogen bonds simultaneously before it can penetrate into the membrane. This involves a large amount of energy; consequently resistance (1) is so high for glycerol that resistances (2) and (3) are dwarfed into insignificance. On the other hand, a molecule like methyl alcohol, having one OH group and one CH_3 group, can easily penetrate into the membrane and can also easily pass out of the lipid layer into water. Thus we find that for such molecules the rate of diffusion across the interfaces is large compared with the rate of diffusion across the membrane, i.e. the viscous resistance (3) of the interior of the membrane is the most important factor in controlling penetration. Differing from both of these examples are predominantly hydrocarbon molecules, such as carotene, $\text{C}_{40}\text{H}_{56}$. For such a molecule resistance (1) is insignificant, for the molecule has no polar group and even resistance (3) is not enormous, but resistance (2) is very large indeed. This is because hydrocarbon groups are hydrophobic and a considerable amount of kinetic energy is required to transfer a CH_2 group from lipid into water: when many such groups are present they must all be transferred simultaneously from the lipid layer into water, since otherwise the molecule remains substantially part of the lipid layer and cannot diffuse away into the aqueous phase. So when a molecule contains many non-polar groups resistance (2) becomes of major importance. With these examples in mind we can classify penetrating molecules into four groups:

- (a) Molecules having few polar and few non-polar groups: for these resistance (3) is most important; they usually penetrate comparatively rapidly, e.g. oxygen, methyl alcohol.
- (b) Molecules having a predominantly polar character: resistance (1) is most important; penetration is slow, e.g. glycerol, sugars, glycogen.

- (c) Molecules having few polar and many non-polar groups: resistance (2) is the most important; penetration is slow, e.g. carotene, vitamin A, cholesterol, fats.
- (d) Molecules having many polar and many non-polar groups: resistances (1) and (2) are both important; penetration is slow, e.g. polyhydroxylic bile acids, the glucuronide of oestrin, proteins.

These relationships between different molecular species and the cell membrane are of great importance, both for the life of a single cell and for the life of the larger organisms. Let us consider just a few of these applications. Oxygen is required in large amounts by the cell and CO₂ must be disposed of rapidly. These substances penetrate the cell membrane rapidly. On the other hand, the first products of glucose utilization by, for example, a contracting muscle cell, such as glycerol derivatives, are valuable: the cell membrane does not allow these to escape. But during sustained work lactic acid is formed: this would be toxic if allowed to accumulate, but the cell membrane is comparatively permeable to lactate, so that lactic acid can escape into the blood. This allows violent exercise to be maintained for a period perhaps 50 per cent. longer than would be the case if the cell membrane were impermeable to lactic acid. Turning then to substances to which the cell membrane is impermeable, we find the problem of storage of vital materials is solved by the utilization of the properties of certain types of molecules—amino-acids are stored as protein, fatty acids as neutral fat, and sugar as glycogen; in each case, as a result of polymerization, a substance is formed which cannot penetrate the cell membrane. An alternative use of inability to penetrate the cell membrane is seen in detoxication mechanisms, in which toxic substances are conjugated with amino-acids, or with sulphuric acid or glucuronic acid, so that a toxic cell-penetrating substance such as brombenzene or menthol is converted into a new molecule which cannot penetrate into cells and which, once reaching the blood-stream, is bound to be filtered off by the glomerulus of the kidney, and will not be resorbed from the urine in the tubules unless it happens to fit into the rather specific secretory mechanism of the tubules. Thus by conjugation with a molecule such as glucuronic acid, two objects are secured simultaneously: (a) the toxic substance is prevented from penetrating into cells, and (b) its excretion into the urine is secured.

Turning now to consider very briefly the problems of secretion, it is possible that at least two seats of activity can be distinguished, the cell surface and the cell interior. Many theoretical proposals have been put forward, involving one or both of these sites, by which molecules such as sugar and ions such as amino-acids and potassium may be moved against a concentration gradient. But the most that can be said for these proposals is that, whilst they are theoretically possible, very few

of the assumptions have yet been subjected to experimental examination. Experimental work on the mechanism of secretion is scanty and mostly of two types, either exploration of the mechanical factors involved or the use of enzyme poisons to see how the source of energy may be cut off. As an example of the first type we may consider the contractile vacuole: it is possible that all the work is being done during the swelling phase of the vacuole, in which unwanted substances are collected from the cytoplasm and separated from other cytoplasmic constituents. Ejection of fluid may result mechanically from the vacuole coming into contact with the plasma membrane; since the vacuole has a higher curvature than the plasma membrane it will have a higher internal pressure than the rest of the cytoplasm if both membranes have the same tension at the surface; consequently, if the vacuole membrane fuses at any point with the cell membrane and the cell membrane cannot achieve the same curvature as the vacuole membrane, the excess internal pressure will force the vacuole contents out into the medium, just as, in the case discussed under 'Wetting Properties', an oil drop of sufficiently high surface tension may be forced into the interior of a cell. Enzyme poisons such as cyanide, fluoride, iodoacetate and phloridzin are frequently used in the study of secretion with the object of finding the particular enzyme systems concerned. But at present it is very difficult to avoid the argument that such poisons may block secretion by interfering with the metabolism at a point far removed from the secretory apparatus. By analogy one may consider the investigation of the process by which an electric lamp produces light; one may cut off the light by dropping a bomb on the power station or by removing the filament from the lamp. At present we can only drop the bomb, whereas what we need to do is to examine the filament.

h. The Ectoplasmic Layer.

In his recent book on the cell surface Just⁶⁰ gives a stimulating account of much of what is known of the ectoplasm of the cell. Although this is achieved at the expense of largely ignoring the cell membrane, Just has performed a timely service in his discussion of a field that has of recent years been somewhat neglected. By the ectoplasm we refer to the cytoplasmic region lying immediately inside the plasma membrane. Its most prominent characteristics are its gelled condition and its relative freedom from the various cytoplasmic formed bodies which constitute a large part of the more fluid, more central endoplasmic region. The ectoplasm is not to be confused with other gel-like structures outside the plasma membrane: these can usually be washed away, or dissected away, without grave consequences. The ectoplasm, on the other hand, is an essential part of the cytoplasm, and its partial differentiation from the endoplasm is partly due to the presence of

the plasma membrane. In most cells the ectoplasm or cortex is a relatively rigid gel and is comparatively clear. It may be liquefied reversibly. For example, Heilbrunn and Daugherty⁵³ found that K^+ decreases the viscosity, or rather rigidity, of the ectoplasm of *Amoebae* and at the same time increases the viscosity of the endoplasm. The reverse action is found with Ca^{++} and Mg^{++} . These observations suggest that K^+ partly releases a gel-forming substance from the cortex and that the divalent ions cause it to be more concentrated in the cortex. Heilbrunn believes the substance involved is ionic calcium. It seems more probable to the present writer that the action of the ions is to lower (in the case of K^+) or raise (in the case of Ca^{++}) the value of the resting potential across the plasma membrane, and that this decreases or increases the rigidity of the gel by decreasing or increasing the orienting action of the cell membrane on the myosin-type molecules involved in gel formation. Marsland and Brown⁷¹ found that high hydrostatic pressure will also reversibly liquefy the ectoplasmic gel. Just suggests that this clear gel is substantially identical with the clear ground-substance of the endoplasm, the differences in physical properties and behaviour being comparatively minor. This suggestion seems quite plausible when it is remembered that the endoplasm also is the site of continuous gel \rightleftharpoons sol changes, and it is possible that the main distinction between the ectoplasm and the endoplasmic ground-substance lies in the localization of more gel-forming material in the ectoplasm—a localization which may be due entirely to the cell membrane. This continuity of structure is rendered the more probable by the absence of any clear division between ectoplasm and endoplasm: the two appear to merge in most cells by quite imperceptible stages, the gel simply becoming weaker at greater distances from the cell membrane.

Sol \rightleftharpoons gel transformations in the ectoplasm are most important in amoeboid activity, in cell division and probably in protoplasmic streaming. It is also possible that the formation of connective tissue fibres, cartilage and bone is an activity of the ectoplasm.* The intercellular bridges known to occur in plants, and believed to occur in animal tissues, are probably mainly ectoplasm. The fine tentacles and filaments thrown out from the surfaces of many cells owe their stability to the rigidity of the ectoplasm. The ceaseless streaming movements of the plasma membrane, so beautifully shown by Chambers' films of marine ova, may be due to the movements of the ectoplasm. Just suggests that muscle cells consist mainly of ectoplasm and that it is to

* X-ray studies (see review, Picken⁵³) show that many tissue fibres consist of parallel cross-linked polypeptide chains. Astbury has shown that somewhat similar fibres may be made artificially from protein monolayers. Hence it is worth considering whether these fibres are not spun off from the adsorbed film of protein present at the surface of cells.

this that muscle owes its high contractile power. Mirsky,⁷⁴ from studies on isolated myosin, has concluded that the myosin in muscle must be in a gelled condition.

Various types of further differentiation of the ectoplasm are found in special cases; for example, the brush border of intestinal cells, flagella, cilia, contractile vacuoles, &c. Wherever visible movements occur, the ectoplasmic gel, or some analogous gelled structure, such as the mitotic spindle, is involved. How far the organized molecular movements involved in secretion are made possible by gel structures remains to be seen.

i. The Physiological Response to a Stimulus.

The cell membrane is not merely a boundary between the cytoplasm and the environment and a convenient support to some enzyme systems. It is also the receptor system for all stimuli which are not in the form of radiant energy, coming from the environment or from other cells (really also part of the environment). It is also the nearest point of approach to other cells, so that it is from the cell membrane that stimuli are given out to other cells. At, for example, a synaptic junction of two nerves it is the adjacency of the membranes of two nerve cells, the property of one membrane to emit acetylcholine and of the other membrane to respond to acetylcholine, which enables a stimulus to be conveyed from one cell to the next. The nature of the response to a stimulus is therefore a matter of great importance. R. S. Lillie^{62,65} has discussed the possibility that the initial response to a stimulus is an increase in permeability, applying this theory very widely, from the response of a nerve to electrical excitation to the response of an egg to sperm entry. In the case of nerve Lillie's theory is now known to be substantially correct. There is a potential difference between the two sides of a resting nerve membrane—the resting potential—which may be reduced by an artificial potential difference applied in the opposite direction. According to Lillie's theory, when this reduction is sufficiently substantial, the permeability of the nerve membrane is increased; adjacent charged regions of the membrane are then able to discharge through the more permeable area, as a result of which the newly discharged region becomes more permeable; thus a region still further away from the initial point of discharge is enabled to discharge through this newly permeable region. This process, under appropriate conditions, should continue indefinitely, each region losing its charge by discharge through a more permeable region slightly nearer to the site of the initial stimulus. So the response to a stimulus consists in a discharge of the resting potential followed by an increase in permeability: the conduction of an impulse consists of a wave of discharge, followed by a wave of increased permeability. And the recovery process, from the

physical point of view, consists in the restoration of the membrane potential and permeability to their initial values. That discharge of the resting potential does accompany transmission of the impulse was, of course, known for many years and was one of the main facts on which Lillie based his theory. A. V. Hill and his colleagues measured the heat evolution accompanying transmission of the impulse, finding it to be so very low that it is hardly possible for the impulse to involve more than a minor physical change in the membrane.* Hodgkin⁵⁶ was able to demonstrate that the local electrical currents postulated by Lillie's discharging process do actually exist, and Cole and Curtis¹³ have shown that a large increase in permeability does follow closely the discharge of the resting potential. On the other hand, even after the increase in permeability has reached its maximum, the nerve membrane retains a very considerable resistance, so that only minor changes in structure can have occurred.

Turning to the other extreme, the response of eggs to activation, Lillie was able to show that following activation, either by sperm or by parthenogenetic reagents, *Arbacia* eggs show an increase in permeability to water. On the other hand, Shapiro, for example, has shown that fertilization causes very little change in the permeability of *Chaetopterus* eggs and Lillie found no change in the permeability of *Asterias* eggs after fertilization, so that if a permeability change is really responsible for initiating development, it must be comparatively transient. But Gray has pointed out that in artificial parthenogenesis it may be necessary to expose the egg to the activating agent for a considerable period, so that it may be necessary to maintain a condition of increased permeability for much longer than is the case with nerve and muscle for the stimulus to be effective. Lillie's theory, in its more extreme forms, must probably be rejected, in so far that stimulation of the ectoplasm may probably occur without a change in permeability, but in considering the immediate response of a cell to most forms of stimulation the evidence strongly favours his view that a change in permeability is of fundamental importance. Consequently one of the great problems confronting those who work on the cell surface is to discover the molecular mechanism whereby these reversible changes in permeability may occur.

Heilbrunn⁵² has advanced a second theory of some importance, not necessarily in contradiction with that of Lillie, but rather intended to show how the stimulus, once received by the cell surface, causes a response which, as in the case of the muscle fibre, may involve the bulk of the cytoplasm. According to Heilbrunn, a stimulus causes release of calcium ions which had hitherto been bound to the proteins and lipoids of the cell surface or the cortical gel layer, and this released calcium

* The recovery heat, evolved long after the impulse has passed, is much larger and does involve chemical processes.

initiates the further activity of the cytoplasm. Heilbrunn and his colleagues, particularly Mazia, have brought together a considerable mass of evidence to show that there is an increase in the ionic (ultra-filterable) calcium after stimulation. Unfortunately it is extremely difficult experimentally to distinguish between calcium released in *immediate response* to a stimulus, and calcium released as a *result* of activity initiated directly by the stimulus without the intervention of calcium ions. However, it seems far more probable, to the present writer, that calcium is released rather late in the process of response. Calcium ions can be bound, either in the ionic or non-ionic state, by combination with anions on the surfaces of colloidal molecules, such as proteins. They can therefore only be released by (a) the production of another cation which is preferentially bound, (b) by the production of acid (or some equivalent process)* which will reduce the number of ionizing acidic groups on the surface of the colloids, or (c) by the conversion of the colloids into smaller molecular units, for example, a protein into amino acids. Consequently a stimulus, to release calcium, must first supply either alternative cations for binding, acid or its equivalent, or else initiate the degradation of colloidal particles. So that the chemical event of increase in the free calcium concentration can hardly be one of the earlier events in response: it is much more likely that it is in most cases a consequence of the production of acid (CO_2 , lactic acid) which is the invariable result of cellular activity.

These theories of Lillie and Heilbrunn are the only ones for which sufficient experimental evidence has been adduced to be taken seriously, and, as we have just seen, Lillie's theory is correct in certain cases, but the underlying molecular mechanisms are unknown; whilst Heilbrunn's theory has less evidence in its favour, and again the molecular mechanisms involved by such a theory have hardly been considered. It may therefore be said that the response of cytoplasm to a stimulus is still, on both the physiological and chemical levels, a most intriguing mystery.

Many workers, both biologists and chemists,† have felt that the co-ordinated activity of the cytoplasm demands the existence of a cytoplasmic skeleton, capable of linking the bulk of the cytoplasm with the cell surface and the nucleus. But all attempts to find this cytoplasmic skeleton have failed, and evidence has steadily accumulated to show that the cytoplasm is fluid and usually of low viscosity (see Chapter II). We are thus in a position somewhat analogous to those nineteenth-

* The same result would be achieved by, e.g., decarboxylation of a protein, or intramolecular rearrangement in a protein molecule which resulted in bringing adjacent carboxyl groups closer together, reducing the probability of dissociation of the carboxyl groups.

† See R. A. Peters's lecture, *J. State Medicine*, 37 (1929), as an example of the latter.

century physicists who sought in vain for a material and ponderable ether. But in the last few years a new possibility has appeared, as a result of studies made on gels and sols of tobacco mosaic virus and on gels of certain other materials, such as bentonite. As was pointed out in Chapter II, there are long-range electrical forces operating between large colloidal molecules. These forces are responsible for orienting long, needle-shaped protein molecules parallel with one another, and for some types of gel-formation. Thus undisturbed cytoplasm may have a structure based on orientation of molecules, without physical contact being necessary between the molecules concerned; the cell membrane, nuclear membrane, and other semi-permanent structures in the cell form an initial base with respect to which orientation may be reconstituted after disturbance. It is well known, for example, that the ectoplasmic layer is frequently gelled and is much more rigid than the regions further away from the membrane. The stability of this gelled layer almost certainly depends on the state of the cell membrane, and there is no apparent reason why this orientation pattern based on the cell membrane should not extend into the bulk of the more fluid cytoplasm. It must be emphasized, however, that this is speculation and at present we can do no more than consider the possibilities of such a theory. One of the most attractive of these possibilities is found in connexion with muscular contraction. Birefringence studies show us that in a resting muscle-fibre the myosin molecules (the major protein constituent) are partly arranged as rodlets whose long axis is parallel to the long axis of the fibre. As a result of stimulation, the resting potential across the fibre membrane is greatly decreased and subsequently the fibre develops a tension; simultaneously the orientation falls off. How the fall in resting potential leads to contraction is unknown. But the conception of orientation in an electrostatic field offers a possible mechanism: let us assume that the myosin molecules nearest to the cell membrane are given a definite orientation by the potential across the membrane; since the fibre membrane has a large curvature except along its long axis, the orientation will be parallel to the long axis. These oriented molecules will then constrain other molecules to orientate in the same direction. The stability of this orientated system then depends entirely on the membrane potential, and if this is discharged the myosin will tend to revert to a state of more random orientation. As Meyer has pointed out, such a loss of orientation will be attended by decrease in length, i.e. by contraction. The process of contraction may, therefore, simply mean that, when the constraining influence of the membrane potential is removed, the molecules revert to a more probable state of orientation. As a result of such a reversion, heat will be evolved for just the same reason that stretched rubber, on contracting, evolves heat. The restoration of the resting potential then becomes an essential pre-

condition for restoration of orientation, i.e. for relaxation.* However, whilst it is most desirable that the possibilities of electrostatic forces acting on colloidal molecules should be investigated as far as possible, it is unlikely that our first guesses about the role of these forces will be quite correct. Moreover, in the processes we have just considered we have still not covered the hiatus between the act of contraction and the chemical events involved. But at least it can be said that some such trigger mechanism as that just described does exist in muscle and that the cell surface is a very important part of this mechanism.

Equally provocative is the response of cells to drugs such as adrenaline, histamine, and acetylcholine. Ability to respond to low concentrations of these drugs is relatively specific, restricted to certain types of cell. The response in many cases is elicited so rapidly that it is almost certain that it is the membrane itself which is the receptor system, for insufficient time elapses after application for a significant amount of the drug to diffuse into the interior of the cells. So there is little doubt that the surfaces of certain types of cells must be specifically adapted to certain drugs. At present we cannot say of what that adaptation consists. But it is probable that consideration of the structural peculiarities of different drugs will give us a clue. Consider adrenaline: like the great majority of substances which act on the cell surface, it is a base and consequently, as the cell surface is negatively charged, it will tend, for purely electrostatic reasons, to be in higher concentration at the cell surface than elsewhere. Secondly, it is partly hydrocarbon in character; this again will cause it to accumulate at the oil-water interface of the cell membrane. Thirdly, it has three hydroxyl groups in close proximity to an aromatic (benzene) ring; the investigations of Schulman and Rideal suggest that this will enable the molecule to interact with the

* Whilst the first step in contraction may well be breakdown of orientation and organization due directly to the transient decline of the resting potential, the myosin molecules may actually pass through a much more complicated cycle of events than simply disorientation and reorientation. Possibly changes in the myosin micelles occur. Possibly, as Bernal has suggested, the orientated micelles in a resting muscle have a loose packing (analogous perhaps to tobacco mosaic virus gels) and in contraction a closer packing (possibly analogous to the virus crystals). To some extent supporting this view are the observations of Mirsky⁷² on frog and rabbit limb muscle and of Danielli¹⁸ on crab limb muscle, in which it was found that a decrease in myosin solubility occurs after extensive periods of contraction or in rigor. Mirsky showed that these changes may also be produced in myosin preparations *in vitro* and are not analogous to denaturation by heat. Then there is the further question of the rôle of the chemical events involved in muscular activity: at present we do not know even which of the chemical changes known to occur are due to restoration of the resting potential and which changes participate more directly in the contractile process.

A change in solubility of a myosin-type protein also occurs after fertilization of *Arbacia* eggs (Mirsky), suggesting that this protein is involved in the mechanism of cell division and mitosis.

monolayer of protein adsorbed on the surface of the cell membrane; this is a third factor increasing the relative concentration of adrenaline at the cell surface. Now any one of these factors taken alone would be insufficient to cause a large increase in adrenaline concentration at a cell surface, but all three factors operating simultaneously may have a remarkably large effect. For drugs of the type we have been discussing it is possible to calculate that, with all three factors operating simultaneously, the concentration at the surface of a cell may rise to between 10^2 and 10^8 times that in the bathing fluid. For example, it is theoretically possible that when a cell is bathed by a solution containing 1 in 10^7 adrenaline the concentration at the surface of the cell is of the order of 1 in 100. Thus, by consideration of these three mechanisms of adsorption on the cell surface, we find that drugs, present in bulk in minute amounts, may at a suitable surface be present in very substantial amounts. Turning to consider the specificity of the reaction of the cell surface to certain drugs, we are at once confronted with the possibility that the specificity may reside in the steric arrangements at the cell surface, for only if the molecular pattern of the cell surface is exactly correct, and presents its charged groups, oil surface and protein grouping all in a correct spatial arrangement will all three mechanisms of adsorption be able to act simultaneously. In terms of such a conception it is easy to see that even minor alterations in molecular structure, such as addition of CH_2 groups, or displacement of NH_2 groups along a hydrocarbon chain, will profoundly affect the possibility of specific adsorption at the cell surface.

When we turn to the result of adsorption of drugs on the cell surface, the first thing to consider is the effect such adsorption will have on the permeability of the cell membrane to potassium and on the magnitude of the resting potential, for the excitability of cells appears, in the vast majority of cases, to be irrevocably bound up with this potential. We know from the work of many investigators, such as Höber, Netter, Osterhout, and Cowan,¹⁵ that when there is no potassium gradient across the cell membrane there is no resting potential, excitability is lost, and in at least one case (plant cells, Blinks³) the permeability of the cell to ions is greatly increased. Solandt has shown that under these conditions the heat production of muscle is many times greater than under normal conditions. It is evident that the cell surface is a very labile system, and that if adsorbed drugs are able to produce even minor changes in the packing and orientation of the membrane molecules, the excitability of the cell will be profoundly affected.

Similarly, the changes in protoplasmic viscosity following stimulation of cells by electric currents or ultraviolet light, or by changing the ionic environment (Heilbrunn and Daugherty⁵³), are probably closely connected in most cases with the condition of the cell membrane. Potas-

sium solutions, for example, cause liquefaction of the cortical gel layer of *Amoeba dubia*, whereas calcium solutions increase the rigidity of the gel layer. From what we know of the actions of potassium and calcium on the resting potentials of other cells, it is quite possible that liquefaction of the cortical gel is due to discharge of the resting potential, and increased rigidity to increase of the resting potential.

iii. FERTILIZATION, ARTIFICIAL PARTHENOGENESIS, CELL DIVISION, AND PROTOPLASMIC STREAMING

In the process and results of fertilization some of the various functions of the cell surface, the cell membrane and the ectoplasm appear particularly clearly. In the first place, in many cases (sea-urchins and *Nereis*) attachment of the sperm involves an agglutination-type interaction between the sperm surface and the egg surface, due to the presence of a substance on the egg surface which F. R. Lillie has called fertilizin. Fertilizin is soluble in water and can readily be washed out of fresh ripe unfertilized eggs. A drop of fertilizin solution will cause agglutination of a sperm suspension. After repeated washing sea-urchin eggs may no longer be fertilizable, and fertilizin can then no longer be detected in the final wash-waters. On the other hand, attempts to show the existence of fertilizin in certain other forms have failed, so that it is doubtful whether this agglutination-type reaction is of major importance. But there is no doubt that it does facilitate fertilization of sea-urchin eggs.

The details of the fertilization process differ considerably from species to species. As an example we shall consider *Arbacia*, partly because it is one of the few forms in which I have had the opportunity of following the details of fertilization with care, partly because it is a common form concerning the behaviour of which there is comparatively little controversy. A short time after insemination with a few spermatozoa, one or more is firmly attached to the cell surface. As the spermatozoon comes close to the plasma membrane, streaming movements in the cell surface may become more pronounced. The tip of the spermatozoon enters the surface and a cloudy appearance sweeps over the surface from the site of sperm attachment. Just states that the cloudiness is in the ectoplasmic layer, but it may equally well be on the surface of the plasma membrane: sudden compression of a protein monolayer produces a very comparable cloudiness. The cloudiness clears up, beginning at the site of sperm entry, in a second wave, and a cone forms on the egg surface tending to engulf the sperm head. Suddenly the sperm head passes inside the cone and the fertilization membrane begins to lift away from the surface in the vicinity of the cone. From this point a wave of membrane separation sweeps round the cell and then for a period of about half a minute the space between the fertilization membrane and the

plasma membrane increases. The fertilization membrane arises from the vitelline membrane, and if the vitelline membrane is removed prior to insemination sperm entry may still occur, but no fertilization membrane appears. Loeb showed that if proteins are added to the sea-water in which insemination occurs the fertilization membrane is not lifted, which strongly suggests that it is elevated by the colloid osmotic pressure of proteins lying between the fertilization and plasma membranes. Chambers suggests that filaments extend between the two membranes: these filaments can readily be torn with a micro-needle.

Prior to the visible elevation of the fertilization membrane, a wave sweeps round the cell from the site of sperm entry, which in some way inhibits the entry of additional spermatozoa. The additional sperm are then usually lifted off by the rising fertilization membrane, but Driesch has shown that removal of the fertilization membrane does not enable additional sperm to enter. Evidently sperm entry induces a fundamental change in the relationship between spermatozoa and the cell surface. Many attempts have been made to show that this is paralleled by permanent physical changes in the cell surface, but so far no great success has attended these attempts: changes in permeability and electrical capacity, for example, are found in some species but not in others. An interesting transient change has been found by Just. If placed in tap-water before insemination, cytolysis of *Nereis limbata* eggs occurs in 3 or 4 minutes. But immediately after the sperm entry, cytolysis occurs in about 25 seconds. This increased susceptibility disappears fairly quickly. A similar increased susceptibility also appears in the eggs of *Arbacia* and of *Echinarachnius*. During this cytolysis the region of greater susceptibility appears to be that from which the fertilization membrane has lifted, as with an egg in which partial elevation of the membrane has occurred, the outflow of cytoplasm never proceeds from a region from which the membrane is not lifted. It remains to be seen whether this increase in susceptibility to cytolysis is a common feature in many species. Polyspermy only occurs with large eggs (with which, as Just remarks, we may suspect that the entry of more than one sperm is due to the large time required for the cortical reaction to spread round the cell), or with small eggs which are stale, or which have been treated with some reagent such as nicotine, high temperature, or acid. Cross-fertilization between different species takes place with varying ease, usually with much less ease than specific fertilization, and is frequently assisted by abnormal treatment of the egg. These observations show us that fertilization is in part specific, in part non-specific, and that the relationship between spermatozoon and egg cell which enables fertilization to occur is not a function of one variable only. Gray pointed out that immunological or chemical views of the fertilization process, such as those of F. R. Lillie^{63,64}, meet grave difficulties in explaining the

non-specific aspects of fertilization, such as cross-fertilization. The view of R. S. Lillie, that the initial action of the spermatozoon is to initiate a change in permeability of the plasma membrane, is capable of explaining the non-specific aspects, but not, unless modified, of explaining the specific aspects of the phenomenon. So far, then, as it is possible to see at the moment, activation by a spermatozoon involves an apparently non-specific trigger mechanism, the operation of which is facilitated if spermatozoon and egg have a specific relationship, which is probably a function of the spatial or chemical organization of their surfaces.

Artificial parthenogenesis may be produced by a great variety of treatments of the egg. It is an effect dependent only on the intactness of the cytoplasm, for E. B. Harvey^{45a} has recently shown that enucleated halves of *Arbacia* eggs when treated by parthenogenetic reagents may form fertilization membranes and divide, reaching a blastomere of some 500 cells before serious abnormalities in development appear. Parthenogenetic reagents may be divided roughly into two groups, though some substances probably fall into both groups. The first group consists of cytolytic agents, such as soap and saponin, whose action is almost exclusively on the cell membrane. The second group consists of treatments or reagents whose action is primarily on the bulk of the cytoplasm, such as hypertonicity and cold. Development may occur without elevation of the fertilization membrane. The action of all these reagents is to cause the production of asters in the cytoplasm: many workers, e.g. Morgan, Wilson, Conklin, Herlant, have shown that frequently very many small asters are produced by these reagents. Some workers, such as Loeb^{66,67}, have concentrated their attention on the action of the various reagents on the cell surface; others, such as Just, have concentrated mainly on the effects on the bulk of the cytoplasm, and regard dehydration as the essential step in activation. Both of these extreme views seem improbable: it is most unlikely that cold and moderate hypertonicity can be regarded as cytolytic agents. And, on the other hand, ultraviolet light, pricking, or small concentrations of fatty acids, all of which are effective parthenogenetic agents, can hardly be regarded as dehydrating agents. A more probable view is that the essential function of parthenogenetic agents (if indeed there is only one essential function) is to promote aster formation.* Asters are gels, probably of myosin-type proteins. Their formation in the cytoplasm can therefore probably be promoted (a) by direct action on the

* The present author does not wish to enter into whether division in parthenogenesis is due mainly or in part to the formation of fresh asters, or whether it is wholly or in part due to the growth and division of the female aster, but simply to emphasize that conditions favouring gelation will promote growth and formation both of new and of pre-existing asters.

cytoplasm, and (b) by stimulation of the cell surface. This gives us the scheme of Fig. 5. Cytolytic agents and pricking must give rise to a change in permeability and this, possibly as a result of decreasing the resting potential, may cause an increase in the probability of gelation in the endoplasm. This process is envisaged as being closely analogous to the increase in endoplasmic viscosity in *Amoeba* following treatment

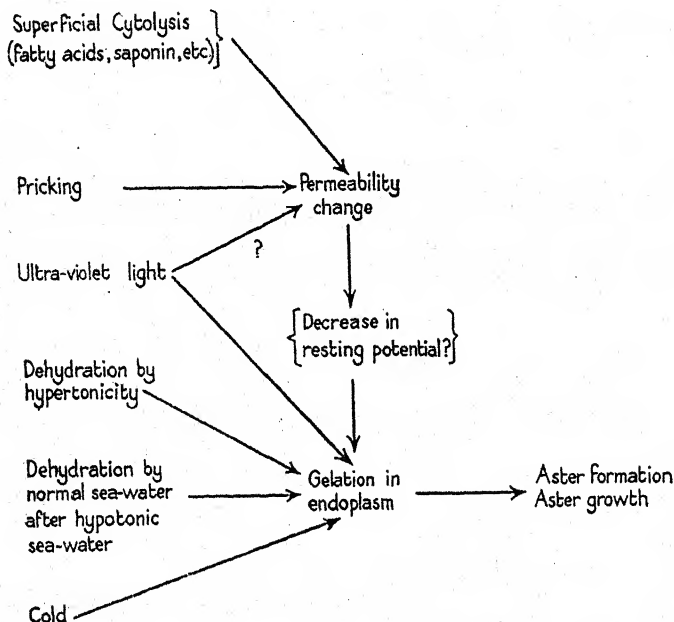


FIG. 5. Diagram illustrating how various types of parthenogenetic reagents may all cause aster growth or aster formation.

with K^+ (Heilbrunn and Daugherty⁵³). Changes in tonicity all involve at some stage a dehydration process: this will favour gelation, as also will low temperatures, without the intervention of a change in permeability. Where excessive changes in tonicity are used, superficial cytolysis may also be involved. Ultraviolet light may act in both ways, as Tchakotine⁹⁵ has shown that ultraviolet radiation has a direct action on the cell membrane as well as on the cytoplasm. Whether this proposed scheme is correct or not, it is certain that the only (visible) initial common feature of the action of the various parthenogenetic reagents is the initiation of gelation and aster-formation. In view of the importance of $sol \rightleftharpoons gel$ changes and amphiaser formation in mitosis and cell division, it seems likely that working backward from this common attribute of the various reagents is more likely to give a clue as to the nature of the mechanism involved than is consideration of the properties of any small group of reagents.

There is no evidence that the cell surface is particularly involved in nuclear division. In cytoplasmic division the ectoplasm at least plays a large part. Originally attention was focused more on the lipid layer of the cell membrane, and changes of surface tension were held by many to be responsible for cell division, but for reasons already discussed it is now certain that surface tension plays at most a very minor role in normal cell division. Recently more attention has been paid to the ectoplasmic or cortical gel layer. Chambers has shown that egg fragments having cortical gel may be fertilized and will divide, whereas fragments lacking cortical gel will not divide. Subsequently further micro-dissection studies have shown that the impingement of the cleavage furrow is due to an invagination of the cortical gel layer: the process is accompanied by increased gel formation, and as the gel invaginates the surface membrane adheres to it and follows the course of the impinging furrow. When the invaginating gel girdle meets in the centre, fusion takes place together with retraction of gel from the small tube still uniting the blastomeres, until finally only a very narrow thread of cytoplasm remains to unite the two cells. This activity of the cortical gel may be inhibited at any time by the application of sufficient hydrostatic pressure to cause liquefaction of the gel (Marsland and Brown; see Chapter II). The action of pressure is reversible in many ways. The protoplasmic thread uniting the blastomeres may persist, possibly indefinitely in some cases, and in others until movements or surface tension forces break the thread. How far similar changes are involved in division of plant cells is unknown.

Many workers have remarked upon the streaming movements of the cytoplasm in dividing cells. Chambers has recently remarked that in cytoplasmic division much of the streaming is closely associated with gel formation in the cortex. It is therefore most interesting that Marsland has found that streaming in *Elodea* cells is associated with gelation, and that streaming is abolished by application of sufficient hydrostatic pressure to liquefy the cortical plasmagel. Marsland suggests that the streaming process is due to a wave of gelation and dissolution of gel passing round the cell and that for example, the fluid is pushed round by the advancing wave of gelation, just as fluid may be pushed along a rubber tube by causing a constriction to pass along the tube. The recent growth of evidence that $\text{sol} \rightleftharpoons \text{gel}$ changes are an essential part of the apparatus of protoplasmic movement is of the greatest importance. Those interested in other potentialities of the plasmagel may consult Needham's book, *Order and Life*.⁸⁰

From the foregoing account it will be realized that rapid and dramatic changes are occurring in our conception of the cell membrane and its relationship with the rest of the cell. The fundamental principles underlying this development, however, are those which were introduced by

such pioneers as Ringer, Overton⁸², Bayliss², Loeb^{66,67}, Morgan^{75,76}, R. S. Höber, and F. R. Lillie^{63,64}, and Hardy. Perhaps it is not too much to hope that the coming decade will see connexions established between this field and the enzymatic processes which furnish the supply of energy for cellular activity. Now that we are approaching the problem of molecular organization in protoplasm, it is reasonable to suppose that we may also make contact with the biochemical processes which, as Hopkins has so lucidly explained, must also be dependent on a complex molecular organization.

CHAPTER IV

MITOCHONDRIA AND GOLGI APPARATUS

By G. BOURNE

i. INTRODUCTION

NO cellular components have aroused more controversy than the mitochondria and the Golgi apparatus and, as a result, any attempt to discuss them is fraught with pitfalls. Since the earlier work on these organelles has been adequately reviewed in 1924 by Cowdry⁴⁰ and in 1931 by MacBride and Hewer¹⁰⁰, reference is made in this chapter mainly to work which has been published since the appearance of these reviews. In the case of the Golgi apparatus there are even more recent summaries of our knowledge; one by Kirkman and Severinghaus in 1938,⁸⁹ and one by Hirsch in 1939.⁶² In consequence only certain aspects of the study of the Golgi apparatus have been dealt with in this chapter. Those who wish for further enlightenment on the problem of neutral red cytology, the role of the Golgi apparatus in male and female germ cells, and other problems not dealt with here should consult these two reviews.

ii. MITOCHONDRIA

a. General.

Mitochondria or chondriosomes are elements of definite form in the cytoplasm of all cells. They lie freely in the cytoplasm, possess the power of independent movement, and may take the form of filaments, of rods, or of granules. Although they were originally described by Altmann⁴ between 1880 and 1890 and were called Altmann's granules, they had, according to Cowdry⁴⁰, been seen and described some years before.

Following the description of these granules there was a period in which various workers showed that many structures thought to be characteristic of cells, including Altmann's granules, could be reproduced at will by the use of different fixatives and staining procedures in models of cells. The result was that Altmann's granule theory was discarded. In 1897, however, Benda demonstrated similar objects in cells without realizing that they were the same as those described by Altmann. He called them 'mitochondria' and was able to demonstrate them in both fixed and living cells.

There are now something like fifty synonymous terms in existence for these structures. The term 'mitochondria,' which has remained in common use, comes from two Greek words meaning respectively 'thread'

and 'granule'. The name is apt because mitochondria are either in the form of filaments or small rods and granules, in the cells of some organs the latter form is typical. In most animal cells the shape of the mitochondria is characteristic for one particular organ. For example, in the adrenal cortex they are in the form of short rods or granules, whereas in the cells of the intestine they are more usually filamentous. They may change in form in secreting glands. Thus in the pancreas they are believed to break up and form granules which eventually metamorphose into zymogen granules. The diameter of mitochondria in individual cells is extraordinarily constant and though they may increase in size, they never do so by increasing their girth, but by an increase of length. Sometimes mitochondria may clump together and coalesce, the bodies so formed are called chondriospheres. Such bodies often occur in scurvy.



TEXT-FIG. 1. Mitochondria in scurvy. Redrawn after Bourne¹⁷.
A. Liver cell; B and C. Kidney cells.

Mitochondria can be seen quite clearly in tissue culture cells under dark ground illumination. Here they appear in a state of constant movement. This movement is of two types: a transposition of the whole mitochondrion from one part of the cell to another (due probably to varying electric charges on the mitochondrial and various cell membranes), and a wriggling movement by the mitochondrion itself. In addition filaments may break up into rods and these may disintegrate further into granules. Granules and rods may join up again and form filaments and the filaments may form temporary networks. Lewis has seen mitochondria in a living cell making a series of journeys from the nucleus to the cell membrane and back again. Mitochondria are frequently clumped around the archoplasmic part of the cell.

According to Policard and Mangenot (quoted by Bensley and Gersh¹³), mitochondria are sensitive to heat and appear to 'melt' suddenly at temperatures of 48° to 50° C., but Bensley and Gersh themselves found that heating frozen dried tissues (see p. 102) to 140° C. for several hours *in vacuo* had no effect on the mitochondria.

Mitochondria seem to be of universal occurrence in living cells. They are well developed even in amoeba. Only in bacteria is their presence doubtful. Wallin¹³³ and others have compared mitochondria to intracellular symbiotic bacteria, but this view is not supported by the majority of cytologists.

Mitochondria are very sensitive indicators of cellular injury. Pinching

of tissues with the forceps before fixation is liable to cause them to break up into granules. Mitochondria do not normally stain with osmium tetroxide (osmic acid) but in injured cells they frequently do, and this may indicate an increase in the amount of unsaturated fat or lipid. In scurvy the mitochondria break up and coalesce (Text-figs. 1, A, B, and C), in beri-beri they do not change. In cyanide poisoning mitochondria maintain their shape, but their movement is inhibited. It may be of interest that mitochondria have been regarded as possessing a respiratory function, and cyanide is known to inhibit certain respiratory enzyme systems.

In the formation of fat and lipid in the animal cell the number of mitochondria decreases. In plant cells it decreases with the formation of plastids. It is claimed by some workers that plastids are formed from the Golgi apparatus, but Guillermond⁵⁸ has provided fairly convincing evidence of their origin from mitochondria. In primitive red blood cells the mitochondria decrease with the appearance of haemoglobin. Various authors have claimed that they are associated with a wide variety of cellular products.

Mitochondria are believed by some to play a part in the formation of yolk in some eggs and to form the middle piece of the mature spermatozoan. (For a detailed review of this subject see MacBride and Hewer¹⁰⁰.) Meves¹⁰⁸ has observed a mingling of male and female mitochondria after fertilization in *Ascaris*, and believes that a conjugation between them takes place. In the developing embryo of the bat the male mitochondria sometimes pass to only one of the first two cells formed by division of the fertilized egg. In some Echinids they have been traced into only one of the cells of a 32-celled embryo. In other cases, e.g. *Nereis*, however, the middle piece of the sperm, which carries the mitochondrial material, does not enter the egg at all.

The measurement of the amount of mitochondria in cells presents difficulties owing to the unavoidable differences in the techniques and the impossibility of complete standardization. Nevertheless, Thurlow¹²⁶ has made an attempt to do so in the nerve cells of white mice. She found that there was a constant number of mitochondria per unit volume of cytoplasm. The cells of some organs, however, may be so different in mitochondrial content from those of other organs that the difference may be seen by simple microscopical observation.

Mitochondria tend to aggregate around the spindle in cell division, and as a result of the pinching in of the cytoplasm they are divided between the two cells in approximately equal quantities. In the case of fibroblasts the mitochondria break up into smaller bodies and become scattered through the body of the cell. Many, however, remain filamentous. Division of the cell body again results in approximately equal distribution (Plate 1, Fig. 2).

b. *Structure and composition of Mitochondria.*

That mitochondria are cell organs of definite shape and structure was demonstrated by Beams and King,¹⁰ who found that in the ultra-centrifuged cells of rat liver the mitochondria maintained their shape and were thrown centrifugally within the cell. The latter fact indicates that they are denser than the cytoplasm.

There is good evidence that mitochondria have a double structure. It may be seen sometimes in mitochondria *in vitro*, and in any case it may become more obvious in large masses of mitochondrial material. The outer sheath or cortex of this double structure from the operation of the Gibbs law probably contains a large amount of fat or lipid. Mitochondria are stained by a method similar to that used for staining *Mycobacterium tuberculosis* and *Mycobacterium leprae*. These bacteria are stained by treating them for some minutes with a hot phenolic solution of basic fuchsin. Once stained in this way they resist the destaining action of acid alcohol. They are known as 'acid fast' bacteria, and their staining idiosyncrasies are believed to be due to the presence of a waxy or lipoidal coat. Mitochondria are stained with hot acid fuchsin and resist the decolorizing action of picric acid for a longer period than most of the other cellular constituents. Up to the time of the development of the freezing-drying method for histological fixation it was believed that most of the substance of mitochondria was soluble in acetic acid, alcohol, ether, chloroform, acetone, and other fat solvents. Mitochondria have a low refractive index which supports the theory of their lipoid nature, and on occasions they may stain with osmium tetroxide. According to Cowdry their smooth outlines suggest myelin bodies.

Much valuable information about the nature of mitochondria has been obtained by Bensley and Gersh¹³ using the freezing-drying method for fixation of tissues. This method was originally introduced by Altman who froze tissue at a temperature of -15° to -20° C. and then dehydrated it in a vacuum. This technique was subsequently improved by Gersh.^{51a} As outlined by Bensley and Gersh¹³ the new technique is as follows. The tissue on removal from the body is frozen immediately in liquid air. It is then placed in a chamber cooled to -20° C., and with the aid of a Hy-vac pump and a mercury vapour pump an initial vacuum of 0.001 mm. of mercury is established, and as the tissue water evaporates the vacuum rises to 0.0001 mm. of mercury. This vacuum is maintained for 12 hours, by which time the drying of the tissues is presumed to be complete. They are then transferred directly into melted paraffin for a few minutes, then embedded, sectioned, and mounted without the use of water. The dehydrating effect of the vacuum depends, according to Gersh, upon the fact that water cooled

to -20°C . has a vapour pressure of 0.9441 mm. of mercury. In a vacuum of 1×10^{-4} the vapour pressure of the ice in the tissue will be much greater than that of such a vacuum, the result will be a rapid evaporation of the water and dehydration of the tissues. The advantages of this technique are, that the solid constituents of the cells are precipitated *in situ* without the extraction and diffusion which occurs as a result of the use of fluid fixatives, and they are precipitated chemically unchanged.

Bensley and Gersh found that the mitochondria of liver cells which had been treated by this technique were unaltered by the application of acetic acid, or by the extraction of the tissue for 50 hours in a Soxhlet apparatus with alcohol, chloroform, acetone, sulphuric ether, or petroleum ether. The same authors point out that it is possible that even these hot fat solvents were insufficient to 'break up a firm protein lipid combination'. Mitochondria have been shown to contain appreciable amounts of fatty material (see p. 108), and since they probably possess an outer adsorbed layer of protein, it is not unlikely that the change in physical state of this protein film, engendered by the freezing-drying process, would make it impermeable to fat solvents. That protein constitutes as well an important part of the 'body' of mitochondria is shown by the fact that these bodies disappear as a result of the activity of proteolytic enzymes (see also p. 120).

It is well known that mitochondria stain specifically with Janus green B (diethylsafraninazodimethylanalin), although Hirsch⁶² claims that this stain also demonstrates the pre-substance of the Golgi apparatus; it also stains certain bacteria. Cowdry³⁹ states that the action of Janus green B is due to the diethylsafranin part of the molecule because a dye of this composition also stains mitochondria specifically. In order to differentiate more clearly between mitochondria and bacteria, Horning⁷⁰ has used a dye, produced by Robertson, which stains mitochondria alone. This dye is Janus red and it is the sodium salt of diethylsafranin monocarboxylic acid.

Robertson¹²⁰ observed that one drop of a saturated solution of safranin added to a solution of trypsin caused the formation of a coloured precipitate. Holtzberg⁶⁹ later demonstrated the proteolytic activity of this precipitate. It was then shown by Marston¹⁰⁴ that other azine dyestuffs including dimethyldiaminotoluazine hydrochloride (neutral red) were also able to precipitate proteolytically active trypsin from solution. When these dyestuffs were added to crude gland extracts the resultant precipitate showed only proteolytic and no lipolytic or diastatic activity. Azine dyestuffs will, however, precipitate a number of proteolytic enzymes, e.g. pepsin, trypsin, erepsin, and papain. The linkage of dye to enzyme takes place, according to Marston, through the basic nitrogen of the heterocyclic azine ring. In view of these

results Marston pointed out that the reaction of mitochondria with Janus green B may indicate that the former contain proteolytic enzymes.

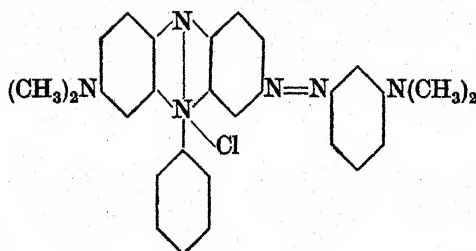
It is of interest that neutral red, which does not stain mitochondria, also precipitates proteolytic enzymes. Hirsch⁶² points out that both neutral red and Janus green B stain what he describes as the 'pre-substance' of the Golgi apparatus. On the other hand, the true Golgi apparatus rarely stains with Janus green, although it may, or at least vacuoles in close association with it may, stain with neutral red. These differences in staining ability suggest—if they are dependent upon the presence of proteolytic enzymes—either that different groups of enzymes are present in the two organs, or that the attachment of the enzyme to, or that the nature of, the substrate is different. It must be remembered, however, that proteolytic enzymes may be distributed in the body of the cytoplasm and the impression that these enzymes are present in the mitochondria or pre-substance may be due to the fact that the chromophoric groups as they are formed become concentrated in these bodies.

Mitochondria gradually reduce Janus green B. Guillermond and Gautheret⁵⁹ point out that this is not a simple reduction. The colour of the dye changes from greenish-blue through red to colourless, and in the red condition the dye may diffuse out into the cytoplasm or into the nucleus or into both. Marston, however, had already recognized that this reduction was not a simple one and had postulated the chemical changes shown opposite. (Reproduced from Marston's paper.)

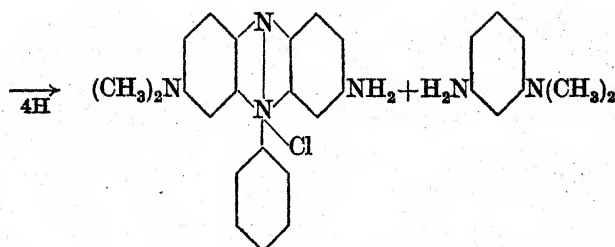
Ludford⁹⁸ found that after the application of a dilute solution of methylene blue to tissue cultures, the mitochondria of the cells stained a brilliant blue. Cytoplasmic granules and vacuoles stained a fainter blue. In some cells after a time there was a diffuse staining of the cytoplasm, but the mitochondria still remained visible because of their more intense blue colour. Ludford found that KCN inhibited the vital staining of the mitochondria with methylene blue. Exposure of the cells to a bright light caused a rapid bleaching of the blue colour of the vitally stained mitochondria. Ludford believes that this may be due to injury of the cell organs which results from intense illumination. The disappearance of the colour may be due to the presence of reducing substances present in the mitochondria, or occurring in them as a result of injury, the action of which is catalysed by the bright light. It is known that reducing substances are present in tissues which reduce methylene blue under the stimulus of light; vitamin C, for example, is one of these substances.

Ludford also found that fewer of the mitochondria of dividing cells stained with the methylene blue than of those of resting cells, and that in less healthy cells the number staining was likewise decreased. Leuco-

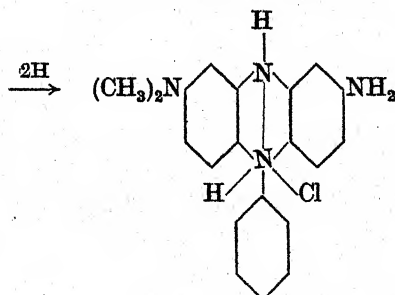
JANUS GREEN



RED DYE



DECOLORIZED DYESTUFF



methylene blue was found to stain the mitochondria very poorly or not at all, but *in vitro* staining was also obtained by the use of toluidine blue and of brilliant cresyl blue. Some staining of the mitochondria was also obtained with methylene blue by the use of the *intra vitam* and the *supra vital* methods, but there was considerable variation of staining by the various cells, and Ludford assumes that this is related to the different oxidation and reduction processes in them. Ludford concludes that 'the staining process is an expression of the vital activity of the cells'. He also points out that the ability to stain mitochondria vitally is not the prerogative of any one dyestuff, since while methylene blue is a thiazine dye and toluidine blue is structurally related to it, brilliant cresyl blue is an oxazine dye.

Mitochondria, although they appear to be of a fatty nature, do not stain with Sudan III; they may be expected to contain protein, but

earlier workers found that they gave a negative reaction with Millon's reagent. Bensley and Gersh¹³, however, believe that this may be due to the quality of the reagent used. They give the formula of a Millon's reagent suitable for use on cells (for details see their paper). Using this reagent Bensley has obtained positive results from the mitochondria of many tissues. Frozen dried sections were found to be particularly suitable for use with the reagent, and Bensley and Gersh obtained positive results with the mitochondria of the cells of *Amblystoma* liver prepared by this method.

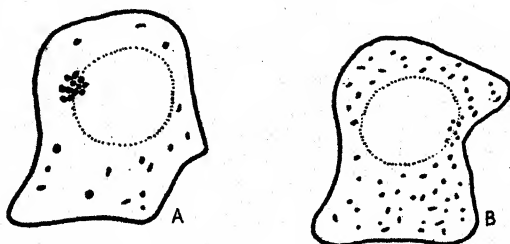
The same authors found that in undenatured, frozen dried sections of *Amblystoma* liver, the mitochondria were destroyed by artificial gastric juice and artificial pancreatic juice (see results of Tarao on effect of proteolytic enzymes on Golgi apparatus, p. 120). They appear to contain no iron or polysaccharides. Gatenby^{50a} has discovered that in *Limnaea* mitochondria are frequently coloured by a yellow pigment (probably a carotenoid). Extracted liver mitochondria also have a yellow appearance.¹² This suggested that small amounts of carotenoids or perhaps of vitamin A might occur in the mitochondria of cells of other animals. It was shown by Bourne¹⁷ and Joyet-Lavergne⁷⁷ that, as a result of the application of antimony trichloride in chloroform solution, the liver cells of mammals showed the mitochondria as bright blue bodies. Joyet-Lavergne has applied this reagent to a wide variety of tissues and animals and claims that in every case the mitochondria show the same reaction. Antimony trichloride is known not to be specific for vitamin A but to give a greenish-blue colour also with carotenoid pigments; therefore a blue reaction on the part of the mitochondria may indicate that they contain either vitamin A, or carotenoid pigments, or both.

It has been shown by Leblond⁵³, Bourne¹⁷, and Giroud and his collaborators⁵³ that mitochondria of some organs react with acetic acid-silver nitrate solution, which is believed to demonstrate vitamin C specifically in histological preparations. This was the case in some cells in the adrenal cortex, medulla (Plate 1, Fig. 4), corpus luteum and anterior pituitary glands (Plate 1, Figs. 7 and 8) of mammals. In some cells the reaction appears in the Golgi apparatus and in others (more rarely) in both.

It has also been found by Bourne¹⁷ and Joyet-Lavergne⁷⁷ that mitochondria frequently give a positive nitroprusside reaction suggesting that they contain glutathione or protein-bound SH (the latter is regarded as most likely by Giroud: personal communication). Joyet-Lavergne⁷⁸ also claims that cadmium lactate reacts with cellular glutathione to form a cadmium glutathione compound which is visible under the microscope. In applying this reagent to a large variety of cells he found that his cadmium glutathione compound was localized particularly in the nucleolus and mitochondria.

Joyet-Lavergne⁷⁸ also claims that mitochondria contain an oxidase system which oxidizes cobaltous salts to cobaltic and that these stain the mitochondria green.

It has been shown by Bourne¹⁸ that the Schultz reaction for cholesterol when applied to the adrenal gland showed the presence, in many of the cortical cells, of small granules and rods strikingly similar to mitochondria. A similar but less intense reaction was shown by the liver, and this mitochondrial reaction did not appear in any other organ treated with Schultz reagent. The absence of a reaction, however, may



TEXT-FIG. 2. Vitamin C preparations of A, cortical, B, medullary cells of rat adrenal, showing mitochondrial impregnation. In A, some granules may also be seen in the region of the Golgi apparatus.

be due to the presence of glycerine, although this may be inhibited if stearic acid is also present (Whitehead^{13a}).

One of the most important advances in the study of the chemical nature of mitochondria has been made by Bensley and Hoerr¹⁴ who extracted mitochondria from liver cells and subjected them to various chemical analyses. These two authors found that mitochondria of the liver of the guinea-pig and rabbit were completely insoluble in 0.85 per cent. saline, and that if a liver emulsion was subjected to differential centrifugation practically pure mitochondrial substance might be obtained. Analyses of such extracted mitochondria showed that 43.6 per cent. of the dry weight was of a fatty nature. Cytologists have always regarded mitochondria as containing a large amount of lecithin or other phospholipid substances. Bensley and Hoerr, however, found that there was no precipitation of lecithin or cephalin when acetone was added to a chloroform solution of mitochondria. The activation-of-cobra-venom-haemolysin test for lecithin was also negative. The test, however, indicated the presence of some free fatty acid. Dried mitochondria reduce osmium tetroxide to a brown colour and the separated fat does likewise; there is thus an unsaturated fatty substance present. Protein is also present in the mitochondria, and while no detailed study has yet been made of the protein fraction, preliminary investigation suggests the presence of two proteins with different isoelectric points.

Subsequently Bensley¹² published the following more accurate composition of dried liver mitochondria (figures approximated).

Proteins and unknowns	65 per cent.
Glycerides	29 " "
Lecithin and cephalin	4 " "
Cholesterol	2 " "

Bensley¹² believes that the surface of mitochondria is composed of a mosaic of protein and lipid micelles. He suggests that the main cortex of the mitochondrion is a mosaic of protein, glyceride, and cholesterol molecules. He believes that the failure of mitochondria to stain with Sudan III is due to the dispersal of the fatty substances. In general Bensley agrees with the suggested structure of mitochondria put forward by Bourne¹⁷, except that he believes the cortex should contain both protein and lipoids* and not only fatty substances as Bourne had suggested.

The presence of cholesterol in Bensley's analysis is a possible confirmation of a histochemical observation (Bourne¹⁸).

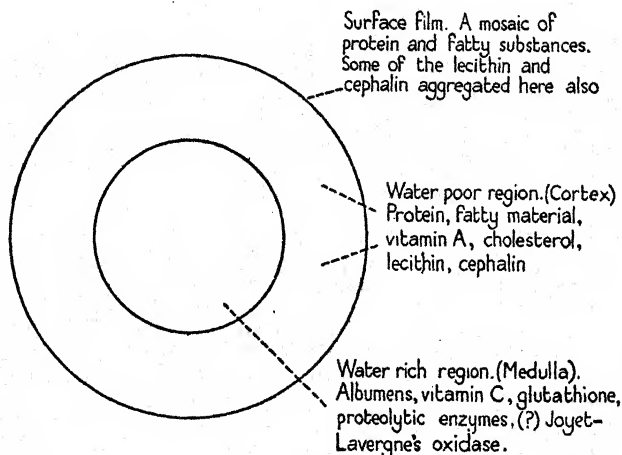
Confirmation of the histochemical test for vitamin A in mitochondria (see Bourne¹⁷, and Joyet-Lavergne⁷⁷) is given by Goerner^{54,55,56}, who, after extracting mitochondria by Bensley and Hoerr's method, showed that 27-32 per cent. of their weight was made up of lipoids which contain vitamin A. He found that 100 mg. of mitochondrial lipid contained 249-910 U.S.P. units of vitamin A. He stated that injection of dibenzanthracene decreased the vitamin A content of the liver mitochondria but increased the total lipid content. He believed that under the influence of dibenzanthracene the liver appeared to lose its ability to split carotene into vitamin A. Goerner found also that the mitochondria of the cells of liver tumours, formed by injecting azo-toluene dyes into animals, contained no vitamin A, whereas those of the surrounding healthy cells contained the vitamin. Further he found that injection of vitamin A increased the vitamin A content of the mitochondria of the healthy cells, but not of the tumour cells. An extension of this line of investigation would probably provide valuable results.

The following is the list of substances which are probably present in liver mitochondria. Some of these (i, ii, iii, iv, and viii) probably occur in all mitochondria; the extent of the occurrence of the others is not known for certain.

- i. Protein.
- ii. Unsaturated (and probably saturated) fatty material (acids and glycerides).
- iii. Lecithin and cephalin (small amount)

* The term lipid is used in a general sense to denote fats, phospholipids, sterols, &c.

- iv. Cholesterol (small amount, probably larger amount in mitochondria of adrenal cortical cells).
- v. Vitamin A and/or carotene. (Liver mitochondria.)
- vi. Vitamin C (probably only in certain endocrine and liver mitochondria).
- vii. Glutathione or protein-bound SH (probably in liver and some endocrine mitochondria).
- viii. Proteolytic enzymes.
- ix. Oxidase capable of oxidizing cobaltous salts to cobaltic salts.



TEXT-FIG. 3. Possible distribution of various constituents of mitochondria.

The water-soluble substances will probably be aggregated in the inner part of the mitochondrion, the fat-soluble substances in the cortex. Protein will probably be present in both parts. See Text-fig. 3. Seifritz has shown, according to Frey-Wyssling⁴⁹, that protoplasmic movement has a pulsating rhythm, and the latter suggests that this may be due to periodic contractions of the polypeptide chains. It seems probable that the wriggling movement of mitochondria which can be seen in living tissue culture cells is also due to the contraction of polypeptide chains of the proteins which are present in them.

Ludford (personal communication) has applied a number of the histochemical tests already mentioned to tissue culture cells without obtaining any positive results for mitochondria. It has been found by Barnett and Bourne (unpublished work) that fibroblasts, except when degenerating and producing fat, show, when stained for vitamin C, only a very small or negative reaction. Furthermore, if vitamin C is added to the medium in which these cells are grown there is no increase in the ability of the cell to stain with the vitamin C reagent (Barnett, unpublished work). If, however, whole chick embryos are stained with the

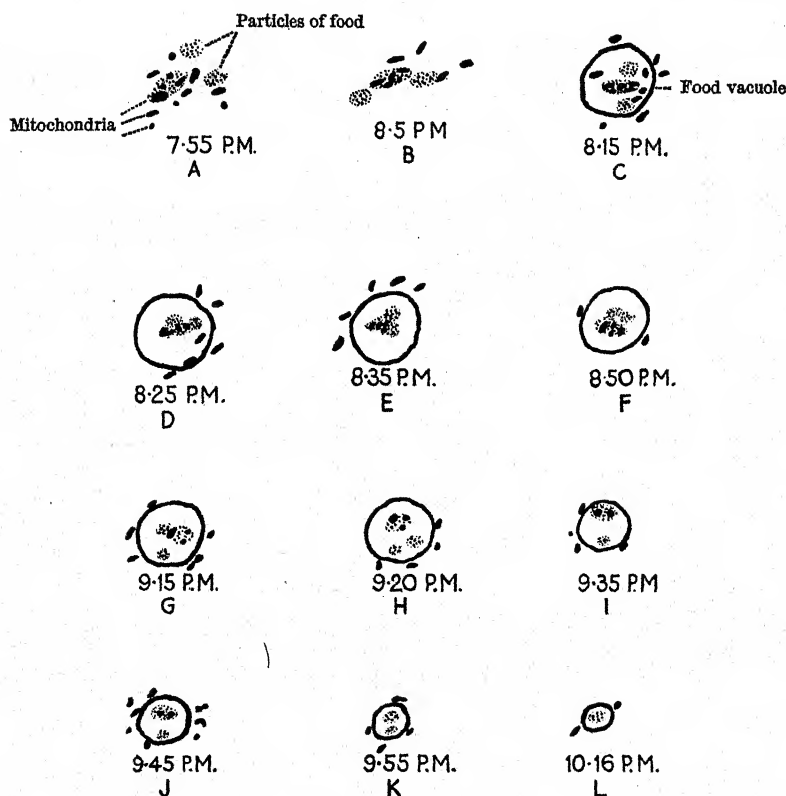
vitamin C reagent many of the fibroblasts give a quite good reaction. If a piece of rat intestine is incubated with vitamin C in normal saline, the connective tissue cells also take up appreciable amounts of the vitamin C. It appears, therefore, that the tissue culture cell is in a different metabolic state from that of the same type of cell in organized tissue. Tests such as the nitroprusside, antimony trichloride, or Schultz do not give a positive reaction with the mitochondria of connective tissue cells, so that these tests together with Ludford's observations suggest that in these cells the mitochondria may not contain glutathione, vitamin A, or cholesterol.

c. Function of Mitochondria.

That mitochondria are the respiratory centres of the cell has been a popular thesis with cytologists for some time. It was first put forward by Kingsbury⁷⁹ in 1912. This hypothesis is based principally upon the fact that fat solvents such as acetone, acetic acids, and alcohol which reduce the respiration rate of cells also dissolve the mitochondria. They also dissolve the Golgi apparatus and probably affect other cell membranes, so there seems no reason why the mitochondria any more than the Golgi apparatus should be regarded as the respiratory centres. A more recent protagonist of the respiratory hypothesis of mitochondrial activity is Joyet-Lavergne⁷⁸. This author finds that mitochondria have considerable oxidizing and reducing power. This he believes to be due to a 'redox' system composed of vitamin A and glutathione. For example, he says that mitochondria oxidize leuco-derivatives of certain dyes and that they can oxidize cobaltous salts to cobaltic salts. He states that he has used the following substances to demonstrate the reducing power of mitochondria: gold chloride, silver nitrate, chromic acid, picric acid, potassium permanganate, *m*-dinitrobenzol. He quotes Chodat and Rouge³³ as having shown that oxidase and peroxidase reactions are given in the region of mitochondria, but he points out that Prenant¹¹⁴, Hollande^{66,67}, and Mangelot¹⁰³ showed that this was so only in certain cells and that the presence of oxidases and peroxidases was not a property of all mitochondria. Although Joyet-Lavergne⁷⁸ believes that the respiratory property of mitochondria is dependent upon a 'redox' system made up of vitamin A and glutathione, there is no evidence that such a system plays a part in any of the known respiratory systems of cells or even that such a 'redox' system exists at all. As direct evidence that mitochondria play a part in respiration he states that young red blood cells of vertebrates have a higher respiration rate, and have more mitochondria, than old red blood cells. So many changes, however, must take place in a red blood cell when it ages that one can hardly claim *per se* that the change in respiration rate is due to the change in the number of mitochondria.

It is quite possible that mitochondria do play an important part in the respiration of the cell, but none of the evidence so far brought forward in favour of this view can be regarded as significant.

Considerable evidence has been brought forward by Horning that



TEXT-FIG. 4. Digestion of food particles by mitochondria in amoeba. Redrawn (modified) from Horning⁷⁰.

mitochondria possess enzyme activity, or rather that such activity occurs at the mitochondrial-cytoplasmic interfaces.

In protozoa, Horning^{70,72,73} has demonstrated both the lytic and synthetic activity of mitochondria.

In amoeba⁷⁰ he found that pieces of engulfed food circulate in the cytoplasm, and that in this process they come in contact with mitochondria which stick to them. A vacuole is then secreted around the food particle and its associated mitochondria. Other mitochondria were found to aggregate on the outside of the vacuole. As digestion proceeds the food particles break up and at the same time the mitochondria become smaller and smaller and gradually pass into solution (Text-fig. 4).

Horning and Petrie⁷⁵ have also found some evidence from the germination of cereals that diastase appears to be secreted in association with the mitochondria. The mitochondria appear to be secreted by the scutellar cells into the starch containing cells of the endosperm; they become applied to the starch grains and result in active hydrolysis. As the starch grains erode the mitochondria disappear.

In the symbiotic protozoan, *Opalina*, Horning⁷³ finds a concentration of 'vegetative' material at the surfaces of the mitochondria. At a certain stage the granules break from the mitochondria and lie free in the cytoplasm (Plate 1, Fig. 3). Horning believes that these granules represent protein material which has been synthesized under the influence of the mitochondria.

Horning is of the opinion that mitochondria are responsible for the production of the zymogen granules of the pancreas and that here, instead of producing enzymes for intracellular digestion, they produce enzymes to be used for extracellular digestion and thus their activity in the lower and higher forms of animal life are fundamentally the same.

d. Relation of Mitochondria to the Golgi Apparatus.

A detailed discussion of this problem is given by Hirsch; it is therefore not necessary to deal fully with it here. It is of interest, however, to quote briefly the views of Hirsch and Ludford on this relationship.

According to Hirsch⁶² there are two groups of views on this question. One group asserts that there is a direct transition of mitochondria into Golgi material, and the other group postulates the liberation by mitochondria into the cytoplasm of various substances which pass to the Golgi apparatus. Ma³⁹, however, believes that the Golgi apparatus is changed in gland cells into secretion granules and mitochondria. Most cytologists, however, do not agree with this view. Other workers have claimed that mitochondria form either the internal or the external part of the Golgi apparatus. Hirsch in general does not favour the view that the mitochondria change into the Golgi apparatus, but the fact that his Golgi 'pre-substance' stains with Janus green B and neutral red suggests that it may be intermediate between the two organelles.

That there is a connexion between mitochondria and Golgi apparatus is shown rather strikingly by Hirsch⁶² on the living pancreas. He found that small granules were formed on the surface of the mitochondria. These granules remained in contact with the mitochondria for a time varying between 10 and 70 minutes, and then they became detached and moved towards the Golgi field at a speed of $1\ \mu$ every 6 to 8 minutes (Plate 2, Fig. 8). Duthie⁴⁵ has seen a somewhat similar picture in fixed preparations of salivary glands and goblet cells of the intestine. Ries¹¹⁸ has confirmed the work on the living pancreas. There is evidence that in other cells mitochondria give off material which is absorbed by the

Golgi apparatus. Ludford^{96a} has stated 'At the mitochondrial-cytoplasmic surface syntheses by enzymes occur. The resulting products continually diffuse into the cytoplasm preventing an accumulation at the surface of the mitochondria which would inhibit further synthesis. At the surface of the Golgi apparatus the elaborated products are concentrated into droplets preliminary to their elimination.' Hirsch⁶² sums up the problem in a similar fashion with the following statement:

Dennoch stehen beide Systeme in einem nahen physiologischen Zusammenhang; es sei, daß Produkte von Mitochondrien als solche direkt in Golgi-Körper übergehen; es sei, daß Stoffe oder Fermente, welche von den Mitochondrien gebildet worden sind, weitergegeben werden an die Golgi-Systeme um hier eine endgültige Verarbeitung zu erfahren oder bei dem Aufbau von Stoffen in den Golgi-Systemen als Katalysatoren zu wirken. Wie der physiologische Zusammenhang zwischen Mitochondrien und Golgi-Systemen im einzelnen Falle sich abspielt, muß noch exakter experimentell untersucht werden.

e. Summary of Mitochondria.

Mitochondria are cell organelles of variable length but of constant diameter in individual cells.

Liver mitochondria are composed basically of protein, fats, and fatty acids, phospholipins and sterols.

They may contain vitamin A and/or carotene, vitamin C, glutathione, proteolytic enzymes, and oxidases.

The composition of mitochondria appears to vary in different tissues under different conditions and is affected by pathological changes in the cells.

It is likely that mitochondria can be divided into an outer and inner portion, the fatty substances being concentrated in the former, and protein being present in both. It is probable that they are surrounded by an adsorbed layer of protein which prevents them from being stained by fat dyes.

There is no satisfactory evidence that mitochondria play a part in cellular respiration, but they are probably involved in proteolytic and, in the case of germinating seeds, diastatic activity.

iii. GOLGI APPARATUS

a. General.

It is impossible in the space available to analyse the vast amount of controversial literature on the Golgi apparatus. It is even impracticable to mention all the various aspects of the subject. Comprehensive reviews are, however, in existence and have been mentioned in the introduction to this chapter. It is hoped to discuss fairly fully some more recent aspects of the study of the Golgi apparatus and to offer a few

tentative conclusions as to the function of this 'most protean of all cytoplasmic inclusions'.⁸⁹

Cowdry writing in 1924⁴⁰ said of the Golgi apparatus:

Even now, 25 years after its discovery, we can only say that the Golgi apparatus is an area of the cytoplasm frequently (especially in higher forms) of reticular shape, often as large as the nucleus, and sometimes definitely located with regard to cellular polarity. Part of the material of which it is composed is soluble in alcohol, becomes blackened after prolonged treatment with osmic acid, and, after appropriate preliminary fixation, shows marked affinity for silver salts. In addition it may occasionally be stained with resorcin fuchsin, iron haematoxylin, and other dyes, but the word 'apparatus' is unfortunate because it carries with it the idea of a mechanism of rather mechanical type.

It is now more than forty years since Golgi⁵⁷ discovered the internal reticular net in the nerve cells of the barn owl and cat. These nets had actually been observed before Golgi (1898) by Platner, 1885,¹¹³ and Hermann, 1891,⁶⁵ and for the first twenty-five years after their discovery investigation of the Golgi apparatus was almost entirely of a morphological nature. During the last ten or fifteen years, however, a great deal of attention has been paid to its function and composition.

During the early stages of the investigation of the Golgi apparatus the subject was confused by the claim of Holmgren⁶⁸ that it was identical with a system of clear canals which he claimed to have discovered in many cells and which he called the 'trophospongium'. Cajal²⁸ added to the confusion by referring to the Golgi nets as Golgi-Holmgren canals. The general opinion of cytologists now is that there is no connexion between the two systems, and the recent evidence obtained from ultra-centrifugation of cells, which shows that the Holmgren canals become stratified in a different position from the Golgi apparatus, seems to have dealt the theory a death-blow.

Later the vacuome theory complicated the issue. Originally propounded by Accoyer¹ it was elaborated by Parat and Painlevé¹¹¹. The theory was described by MacBride and Hewer¹⁰⁰ as follows:

All animal and plant cells have two, and only two, fundamental but independent morphological elements—the vacuome and the chondriome. The vacuome is an aqueous phase; the chondriome a lipoidal one. The vacuome consists either of isolated vacuoles, or else of a canalicular system. The vacuome stains specifically in neutral red *intra vitam*. From these premises they state that the 'reticular apparatus' of Golgi and the 'trophospongium' of Holmgren (in fact the whole classical Golgi apparatus), are artefacts produced by precipitation of silver or osmium at the surface of, inside or between the vacuoles.

Parat later modified his theory to include the dictyosomes and 'announced that the dictyosomes were scale-like cortices of the

"vacuome" and introduced for them two new names, "lepidochondriosomes" and "lepidosomes". He further stated that the "lepidosomes" were merely modified mitochondria, and introduced still another and third name for them, "chondriome actif"⁵¹. The cell thus contained a 'chondriome' and a 'chondriome actif' (or lepidosome) which was associated with the vacuome. The vacuoles in the animal are homologized with the large intracellular vacuoles of plants principally because, according to the protagonists of the theory, they both stain with neutral red. Since the neutral red vacuoles of the animal cell are believed to be identical with the Golgi apparatus it is important to remember Gatenby's⁵¹ comment that not all neutral red staining bodies are argentophile, whereas the true Golgi apparatus always is. Also neutral red affects the cytoplasm of the cell, causing it to form new vacuoles which according to Hirsch⁶² represent the crinome of Chlopin³². Hirsch⁶² claims that neutral red stains his 'pre-substance' but not the Golgi system. There is some evidence that neutral red vacuoles may sometimes occur in connexion with the Golgi apparatus,* but we can certainly regard the vacuome theory as not proved. This matter has not been discussed fully because adequate reviews of the subject are already available (MacBride and Hewer¹⁰⁰; Kirkman and Severinghaus⁸⁹; and Hirsch⁶²). Gatenby's⁵¹ brilliant analysis of 'Neutral Red Cytology' should be read by every serious student of the cell.

Gatenby stated that some representative of the Golgi apparatus could be found in every vertebrate or invertebrate cell. The work of Bowen²² indicated that the Golgi apparatus existed in plants in the form of osmiophilic platelets. There are no records of its occurrence in algae, fungi, or bacteria. Protozoa are known to possess a Golgi apparatus. It has been specially investigated by Horning⁷⁰⁻⁷³, Richardson and Horning¹¹⁷, and by Kedrowsky^{80,85} in the case of *Opalina*, the intestinal protozoan symbiote of frogs.

b. *Morphology of the Golgi Apparatus.*

Kirkman and Severinghaus⁸⁹ state that the Golgi apparatus has been described at various times as consisting of 'a fibrous reticulum, network, ring, or cylinder, a very irregular fenestrated plate, a more or less incomplete hollow sphere, vesicle, or cup, a collection of small spheres, rodlets and platelets or discs, a series of anastomosing canals, a group of vacuoles, and a differentiated region of homogeneous cytoplasm crossed by irregular interfaces'. The apparatus very frequently occurs as a network, and many authors have questioned the validity of such a structure. Hirsch⁶² denies the existence of most networks and other

* See the results of Covell and Scott³⁷ who observed the slow blackening of neutral red granules in nerve cells of a ventral horn smear as a result of the introduction of osmium tetroxide under the cover glass.

workers have regarded them as staining artefacts. It is of interest that grains of reduced silver seem to be composed fundamentally of a network structure. The granules of silver in a photographic emulsion even under the high power of the ordinary microscope appear to be homogeneous, but when they are examined with an electron microscope, magnifying them by 25,000 diameters, they may be seen to be networks, made up of threads, some of which are only five atoms thick (Plate 2, Fig. 2). The type of network can be varied according to the developer used. Hydroquinone produces coarse threads, whereas metol produces fine threads. With some other developers the network becomes a sort of fluffy mass. The form of the Golgi network also varies according to the time of development and the type of developer used.

In various cells the Golgi apparatus varies considerably in size and shape and is usually well developed in the stage of cytomorphosis and tends to decrease in size as the cell becomes older. In gland cells the apparatus is noticeably large, but it is relatively quite small in muscle cells. Actually the problem of making accurate comparative estimations of the amount of Golgi apparatus is almost insuperable, because, as with mitochondria, even with the most accurate standardization of technique unaccountable variations may occur.

As a general rule in vertebrate somatic cells the Golgi apparatus is in the form of a dense network situated near the nucleus; it may, however, vary according to the physiological state of the cell. The general structure varies in cells of different organs, although the shape is never identical even in the neighbouring cells of the same organ. In developing germ cells the apparatus may be in the form of rods or granules. This is also the condition in which it is found in most invertebrate cells. Horning⁷², however, has found Golgi nets in the cells of *Hydra viridis*.

The extreme variability in the form of the Golgi apparatus makes it likely that the apparatus in life is in a state of constant slow movement. In many cases pseudopodia-like extensions of the apparatus may be seen, e.g. medullary cells of the adrenal gland (see Bourne¹⁶), and the apparatus is never exactly the same even in adjacent cells of the same tissue.

In mitosis the Golgi apparatus usually breaks up into small particles or granules which are distributed more or less evenly through the cytoplasm. Cytoplasmic division causes an approximate halving of the Golgi substance. Ludford⁹⁵ has described five different types of division of the apparatus.

The denial of the existence of the Golgi apparatus has been made from time to time. Strangeways and Canti¹²⁴ have adopted this view because they were unable to detect any signs of the apparatus in living unstained tissue culture cells either by direct or dark ground illumination. Ultraviolet light photographs of living unstained cells have also

shown nothing which suggests the Golgi apparatus. Chambers³⁰ in his micro-dissection studies was unable to find anything which suggested the presence of a membrane or vacuoles in the Golgi region of the cell.

According to Ludford⁹⁸ the reason why no Golgi apparatus can be seen in cultured cells is that 'as some cells spread out the Golgi apparatus is stretched until it fragments and its individual particles become dispersed in the cytoplasm'. Richardson¹¹⁶, however, found a large and compact Golgi apparatus to be present in cultured cells (Plate 2, Fig.3). Ludford⁹⁸ later showed that fragmentation of the apparatus occurred only at certain stages in cultured cells, and that it was because Strange-ways and Canti were examining cells in which the Golgi apparatus was fragmented that they were unable to see any signs of it. Ludford has published photographs of living tissue culture cells stained *in vitro* with methylene blue in which a distinct Golgi area can be seen near the nucleus. This Golgi area has a reticulate structure (Plate 2, Fig. 1). The nucleus is the dark oval figure near the left side of the white mass. The Golgi area is the dark region speckled with white immediately to the right of the nucleus.

Baker (Baker, J. R., *Quart. J. Micr. Sci.*, 1944, 85, 1) in a critical analysis of the literature and technique of demonstrating the Golgi apparatus concludes that the form of the apparatus determined by the usual techniques is not an accurate representation of its structure during life. He believes it to be composed of vacuoles having dense lipid-containing substance, the invested vacuoles being either separate or joined into small groups by investment. Heilbrunn⁶³ has also questioned the Golgi apparatus. In 1934 Beams and King,¹⁰ using a centrifuge designed by Professor J. W. Beams and capable of producing a centrifugal force of 400,000 g., showed that in uterine gland cells of guinea-pigs the Golgi apparatus could be removed from its normal position in the cell. This confirmed that the Golgi apparatus was a definite cell organ, and secondly since it was thrown centripetally that it was less dense than the cytoplasm. Since Beams and King found also that there was no displaced system of canals or vacuoles, their experiments dealt a severe blow to the vacuome hypothesis. Hirsch⁶² points out that the inability to see the apparatus in most living cells is no indication that it is not present, because, he says, it has an identical refractive index with that of cytoplasm. Hirsch claims that it is impossible to see the nucleus in the living cells of the pancreas of the white mouse even under the best conditions (this is denied by Ludford, personal communication), but that nobody would say that the nucleus which is apparent after fixation and staining is an artefact. He also states that the assertion by many workers that the Golgi apparatus is never visible in the living cell is wrong. For, apart from Ludford's observations, van Bergen¹⁵ saw the apparatus in the unstained prostate

gland. Hirsch and Bretschneider⁶² have seen the Golgi apparatus in the living intestine cells of *Ascaris*. Gatenby has seen a Golgi apparatus in the unstained living cells of invertebrates, and Macdougald and Gatenby¹⁰¹ claim to have seen the apparatus in living Purkinje cells of mammals and in spermatocytes. Certain ectosomal granules of *Opalina* are also visible in life and these are regarded as forming part of the Golgi apparatus. The apparatus has also been seen in living eggs, and in some cells it can be brought into view (Hirsch⁶²) by the use of neutral red or trypan blue or other dyes, or by varying the acidity of the medium in which the cell is immersed. The behaviour of the apparatus in gland cells emphasizes that we are dealing with a cellular component whose existence cannot be denied. Kirkman and Severinghaus⁸⁹ state:

The most conclusive proof that the Golgi apparatus is an organelle is not the universality of its presence in properly fixed material, nor even the descriptions of it in living material, but rather its behaviour as determined by microscopical observations of it in the same type of cell in different physiological conditions. This behaviour is so characteristic and so constant that there should be no question concerning the reality of the substance or structure exhibiting it.

That the Golgi apparatus consists of two parts, an outer which absorbs osmium and silver and an osmic- or argento-phobic inner portion, has been found by a number of workers (including Bowen²²; Weier¹³⁴; Richardson¹¹⁶; Uhlenhuth¹³⁰; Bourne¹⁶, Hirsch⁶²). This double structure is beautifully shown in Plate 2, Fig. 3 (taken from Richardson¹¹⁶). Hirsch has found that the Golgi bodies of the cell do not always have this double structure and he has recently devoted a book to a consideration of his pre-substance-Golgi-system theory. Hirsch declares that the solid granules of Golgi substance which show no differentiation into external and internal portions really constitute the 'pre-substance' of the Golgi apparatus. These pre-substances are able to build up nets, but they are the only sort of nets that Hirsch recognizes. The other nets, he claims, are due to over-impregnation of separate bodies by excessive amounts of osmium or silver which causes them to link up and simulate a net. These pre-substances may be aggregated near the nucleus or distributed throughout the cell. Each piece of pre-substance gradually develops a double structure with an argentophile and osmiophile cortex; at this stage it is referred to as a Golgi system. A number of joined Golgi systems are known as polysystems. The outer part or cortex of the Golgi system is known as the externum, the inner part as the internum. Hirsch⁶² believes that the product* of the cell is formed in the internum; he also gives the impression that only the pre-substance stains with neutral red and only the Golgi system with trypan blue.

* The 'product' of the cell is the term used by Hirsch to describe the granules of secretion which are so obvious microscopically in externally secreting glands.

He claims further that the pre-substance stains with Janus green B. A good illustration of Hirsch's results is given by his work on the living pancreas cells of the mouse. He found that the bodies which were in the Golgi field were of two types: first, granula B¹ which were on the lower edge of the field and were coloured by neutral red and Janus green; secondly, granula B² which lay in the upper edge of the Golgi field and gave hardly any neutral red reactions (Plate 2, Fig. 8). These bodies are separate, and according to Hirsch, descriptions of a Golgi net in the acinar cells of the pancreas are incorrect. Granula B² could be impregnated superficially with osmium giving the impression of a double structure and therefore could be regarded as Golgi systems. The fact that granula B¹ stain with Janus green B, which has been regarded as a specific stain for mitochondria, suggests that the granula B¹ (the pre-substance granules) are of mitochondrial origin, or else that like mitochondria they may contain proteolytic enzymes. The former suggestion is supported by the fact that they can be fixed with Flemming's and Regaud's solutions and stained with iron haematoxylin. The zymogen granules of the pancreas are eventually produced from the Golgi systems, but if the pre-substance can be derived from the mitochondria, then these results can be brought into harmony with those of investigators who have found that the zymogen granules come from the mitochondria. Duthie⁴⁶ has obtained somewhat similar results to those of Hirsch in the Harderian gland of the rat.

c. Composition of the Golgi Apparatus.

Nath (quoted by Hirsch⁶²) has stated that the Golgi apparatus can be demonstrated in eggs by treating them with 2 per cent. osmium tetroxide for 10-15 minutes. This capacity on the part of the apparatus to reduce osmium tetroxide even without prior fixation has led many authors to claim that the Golgi apparatus contains unsaturated fats and fatty acids. This is not necessarily so, for the Golgi apparatus may contain vitamin C, which also reduces osmium tetroxide, and Lison^{84a} has pointed out that the latter demonstrates a number of reducing substances. Baker (see p. 117) points out that up till now there has been no reliable evidence as to the chemical nature of the osmiophilic portion of the Golgi apparatus, except that it contains lipoidal substances. But he produces strong evidence from various independent sources that it contains lecithin or cephalin, or both. In some cases fat dyes have been used to stain the Golgi apparatus (Cowdry³⁸; Weiner¹³⁵), but this is rare. The fact that the apparatus is soluble in the usual fat solvents suggests that it contains a high percentage of fat. In the ultracentrifuged cell the Golgi apparatus goes to the centripetal part of the cell, presumably owing to its lipoid nature. Bowen has suggested that protein is present in the apparatus and Gatenby is of the opinion that the

apparatus is a combination of protein and lipid. Ciaccio^{34, 35} has advanced a histolipoid theory suggesting that some of the lipid substances contained in animal cells are in loose combination with protein which prevents them from giving the usual lipid reactions. Ciaccio used 'unmasking' procedures to set free the lipid. In some cases he used proteolytic enzymes. He was able by these methods to demonstrate the idiozomic complex in the male germ cells of *Discoglossus* with fat dyes.

Tarao¹²⁵ applied this unmasking procedure to the hepatic cells of the mouse and the newt. The livers were first fixed in formalin, then frozen sectioned, and then digested with pepsin and trypsin in turn. They were then stained with aqueous Nile blue sulphate which is used normally to stain fats and lipoids. Tarao found that the dye had demonstrated the Golgi apparatus. Two photographs (Plate 2, Figs. 4 and 5) taken from his paper show the similarity between the Golgi apparatus demonstrated by this method (Plate 2, Fig. 5) and that demonstrated in the usual way by Kolatchev's osmium tetroxide method (Plate 2, Fig. 4).

It is of interest that Tanaka^{124a} secured vital staining of the Golgi apparatus of tissue culture cells with Nile blue sulphate.

We may therefore conclude that the Golgi apparatus like the mitochondria is composed of protein and lipoidal or fatty substances, or more probably that like the mitochondria it may possess a superficial adsorbed layer of protein. The fact that the mitochondria do not react with Nile blue sulphate suggests that they have been completely dissolved by the enzymes and that therefore they contain more protein relative to the lipid in their structure than does the Golgi apparatus. The fact that the apparatus is sometimes stained with dyes which are used for demonstrating connective tissue fibres supports Tarao's evidence that it contains protein. Experiments with chemical models have shown that fatty droplets in solution containing protein are always surrounded by a layer of adsorbed protein. It is very likely that such a protein layer is present on the surface of both mitochondria and Golgi apparatus, and that this explains why they do not normally react with fat stains.

In some cells the Golgi material may appear to be a granule; in others it may appear semi-fluid in character. It seems possible that it may vary in consistency from time to time in the same cell; this would not be surprising, for a rearrangement of the protein molecules in the apparatus would rapidly alter the nature of the material. How rapidly this rearrangement of protein molecules can occur in the living cell is illustrated by Seifritz.¹²² If the flowing cytoplasm (at this stage of low viscosity) of *Rhizopus* is pressed with a micro-dissection needle it suddenly becomes quite rigid. The gel so formed spontaneously reverts to the low viscosity liquid condition after a time. A gelation of parts of

the cytoplasm of cells also occurs during the formation of the mitotic spindle.

The association of vitamin C with the Golgi apparatus is a recent conception, and some space will therefore be devoted to a discussion of this matter.

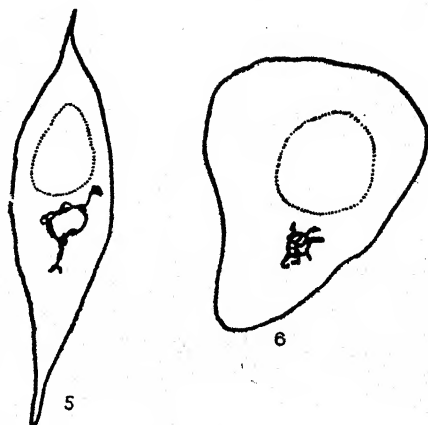
Observations by Bourne^{16,17}, in numerous papers by Giroud and Leblond (see Giroud⁵³), and in a series of papers by Tonutti^{127,128,129} have suggested that in a large number of cells vitamin C may be associated with the Golgi apparatus. In some cells, e.g. those of the adrenal cortex, vitamin C may occur in the mitochondria instead of the Golgi apparatus, and in other cells, but more rarely (Giroud), it may occur in both mitochondria and Golgi apparatus. Vitamin C is demonstrated histologically by means of a solution of silver nitrate in 10 per cent. acetic acid. For recent reviews of the specificity of this reagent see Giroud⁵³ and Barnett and Bourne^{5a}.

During the histogenesis of tissues in chick embryos Barnett and Bourne⁶ observed that in many cells vitamin C appears to be localized in the region of the Golgi apparatus. This is particularly evident in nerve cells (Text-figs. 5 and 6) where, in addition, it may occur along the developing axon. A particularly striking correspondence is shown in Text-figs. 7 and 8, which demonstrate a vitamin C preparation of the liver of a 10-day chick embryo (Text-fig. 8) and a Golgi preparation (Text-fig. 7; Dalton⁴²) of an 11-day chick embryo. In dividing cartilage cells of chick embryos (Text-fig. 11) the disposition of the vitamin C granules around the chromosomes and the spindle figure is strikingly similar to the disposition of the Golgi apparatus in dividing cells. It is of interest that these cartilage cells were the only dividing cells which showed the presence of vitamin C. Other cells even if they contained vitamin C before division appeared to lose it when the time came to divide.

Ries¹¹⁹ has figured the egg of *Aplysia* (Plate 2, Fig. 9) stained to demonstrate granules of vitamin C. It appears that these granules may, according to Hirsch⁶², represent Golgi pre-substance, but he is uncertain about it. When the egg starts to divide, the granules pass to certain cells, while others are left without granules. This is a rather different behaviour from what we believe to be characteristic of the Golgi material in dividing embryonic cells. Very little information is available so far on the distribution of vitamin C in the eggs and early embryos of various animals, and this subject would provide a fertile field for research. (For the distribution of vitamin C in the chick embryo, see Barnett and Bourne^{5a,6}.)

Miwa¹⁰⁹ has shown that the decrease in size of the Golgi apparatus (demonstrated by standard techniques) in the damaged liver of rabbits is similar to the decrease in size shown by the area containing vitamin C

(acetic acid-silver nitrate technique). There was a parallel increase in the Golgi apparatus and the vitamin C staining area in the liver and

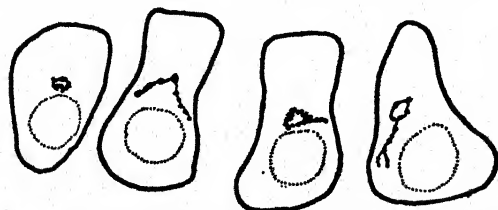


TEXT-FIG. 5. Neurone of developing chick. Golgi preparation. Redrawn after Alexenko³.

TEXT-FIG. 6. Vitamin C preparation of neurone of developing chick. Redrawn after Barnett and Bourne⁶.

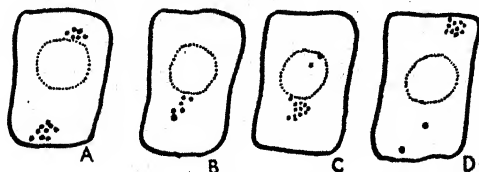


TEXT-FIG. 7. Golgi preparation of 11-day chick embryo liver. Redrawn after Dalton⁴².

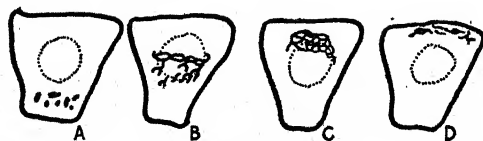


TEXT-FIG. 8. Vitamin C preparation of 10-day chick embryo liver. Redrawn after Barnett and Bourne⁶.

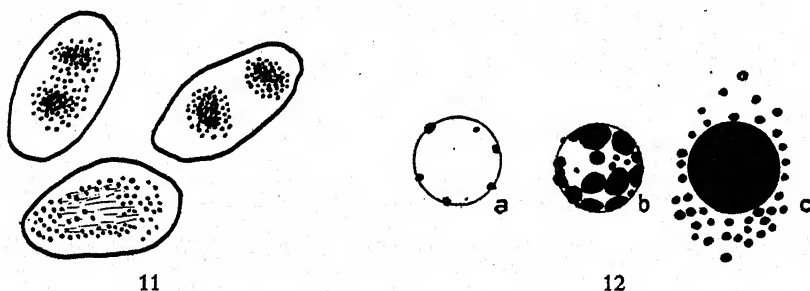
capsule of the rabbit after injection of vitamin C. In scurvy in the marmot, Miwa showed a roughly parallel decrease in Golgi apparatus and vitamin C staining area in kidney and liver cells. Injection of vitamin C into a scorbutic marmot caused a parallel increase in Golgi apparatus and vitamin C staining area in liver capsule and kidney cells. He concludes that vitamin C is associated with the Golgi apparatus.



TEXT-FIG. 9. Vitamin C preparation of 23-day chick metanephros showing apparent passage of vitamin C across the cell.

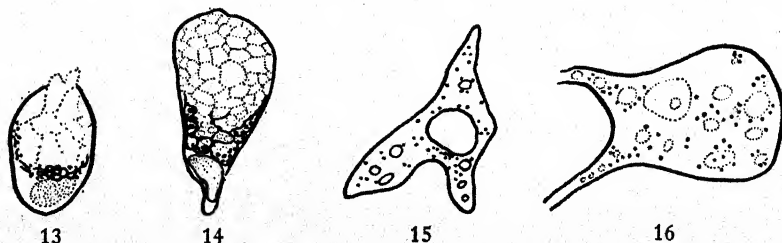


TEXT-FIG. 10. Changes in the position of the Golgi apparatus of the rat's kidney during diuresis. Redrawn (modified) after Fischer⁴⁷.



TEXT-FIG. 11. Golgi-like distribution of vitamin C granules in dividing cartilage cells of developing chick. Redrawn after Barnett and Bourne⁶.

TEXT-FIG. 12. Stages in adsorption of vitamin C on to a 'Golgi system' in the intestinal cells of the starved *Ascaris*. Redrawn after Hirsch⁶².



TEXT-FIG. 13. Vitamin C preparation of goblet cell in rat's ileum.

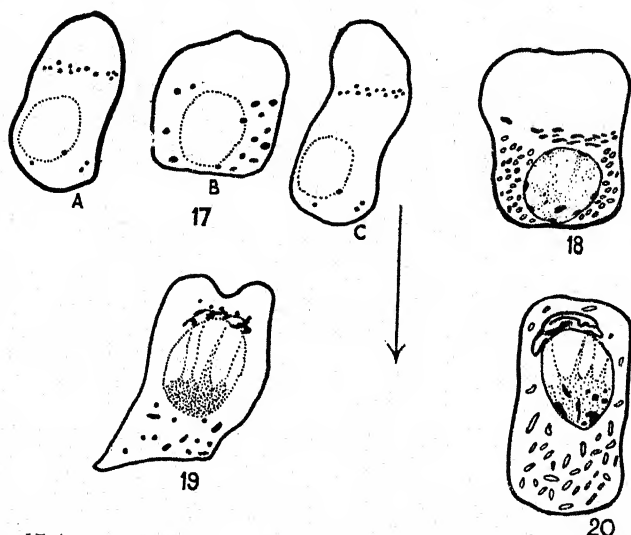
TEXT-FIG. 14. Golgi preparation of goblet cell of rat's colon. Redrawn after Duthie^{44a}.

TEXT-FIG. 15. Distribution of Golgi substance in fibroblast producing fat. Redrawn after Macdougald and Gatenby¹⁰¹.

TEXT-FIG. 16. Distribution of vitamin C granules in fibroblast producing fat.

In Hirsch's laboratory strips of intestine of the starved *Ascaris* were incubated in 0.1 per cent. solution of vitamin C. The cells absorbed the vitamin and concentrated it in the Golgi apparatus (Text-fig. 12). Hirsch suggested that the vitamin C was absorbed by the cells and distributed in the cytoplasm in a finely divided form which was not visible through a microscope, and that it was then concentrated in the Golgi apparatus.

Bourne (unpublished work) has obtained similar results with rats'



TEXT-FIG. 17 (A, B, and C). Ultracentrifuged cortical cells of rat's adrenal stained for vitamin C.

TEXT-FIG. 18. Ultracentrifuged cortical cell of rat's adrenal showing distribution of Golgi material (black rods) and mitochondria (ovals). After Dornfield⁴³.

TEXT-FIG. 19. Ultracentrifuged medullary cell of rat's adrenal, stained to demonstrate vitamin C.

TEXT-FIG. 20. Ultracentrifuged medullary cell of rat's adrenal to show distribution of Golgi material (black network) and mitochondria (rods and ovals). After Dornfield⁴³.

intestines incubated with vitamin C in normal saline. Text-fig. 13 shows a goblet cell from a piece of ileum incubated in this fashion for half an hour and stained for vitamin C. It may be compared with Text-fig. 14, which shows a goblet cell of the rat's colon stained to demonstrate the Golgi apparatus.

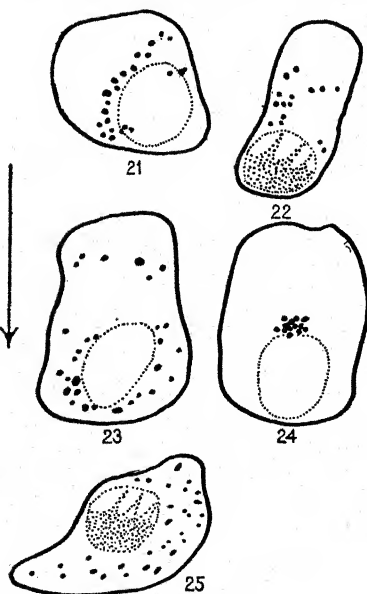
Hirsch⁶² believes that the vitamin C first permeates the externum of the Golgi apparatus, but that finally it passes through it and becomes localized in the internum, where it plays a part in the formation of the 'product' of the Golgi apparatus (Text-fig. 28).

If fibroblasts in culture are stained with the silver nitrate reagent for vitamin C they give practically no reaction. Occasionally a few small granules may be found in the region of the idiozome. Cultured

fibroblasts have, however, a well defined Golgi apparatus (Plate 2, Fig. 3). When a fibroblast is forming fat the Golgi apparatus breaks up into small granules which are scattered through the cytoplasm. These granules are often attached to the fat globules (Text-fig. 15). Vitamin C preparations of cells in this condition show the granules of vitamin C to be distributed in a similar fashion to the granules of Golgi material (Text-fig. 16). (Barnett and Bourne, unpublished work.)

Tonutti¹²⁷ has injected vitamin C together with trypan blue into animals and examined the kidney cells and, in the case of pregnant animals,^{128a} the decidual cells. In each case the trypan blue was localized first in the region of the Golgi apparatus, and staining the tissue for vitamin C showed that in the centre of the blue areas produced by the trypan blue were black granules which indicated the presence of vitamin C (Plate 2, Fig. 10).

It was originally shown by Jasswoin^{76a} and more recently by Ludford^{96a} that trypan blue granules form in the region of the Golgi apparatus and then pass into the body of the cell in the same way as the secretion droplets of gland cells. Since Tonutti has shown that the droplets of dye so formed contain a central granule of vitamin C, it appears likely that the vitamin is adsorbed also on to the Golgi apparatus and then segregated into granules in the same way as the trypan blue. It appears to be by means of granules such as these that vitamin C is passed across the kidney tubule cells. It has recently been shown by Fischer⁴⁷ (Text-fig. 10) that during artificially induced diuresis in the white mouse the Golgi apparatus of the tubule cells shows a periodical reversal of polarity. If this is so, for there is earlier work which denies this change of polarity of the Golgi apparatus during diuresis (see Hirsch⁶²), then it is possible that the Golgi apparatus itself acts as a transporting agent for vitamin C. The important point, however, is that the vitamin does not diffuse freely across the cell but is segregated by the Golgi apparatus and makes its passage across the cell either in the apparatus or in the form of segregated granules.



TEXT-FIGS. 21, 22, 23, 24. Various ultracentrifuged cortical cells of rat's adrenal stained to demonstrate vitamin C.

TEXT-FIG. 25. Ultracentrifuged medullary cell of rat's adrenal stained to demonstrate vitamin C.

The apparent passage of vitamin C through the tubule cells of the embryonic chick metanephros is shown in Text-fig. 9.

When the adrenal gland of a rat is ultracentrifuged at a force of 400,000 g. for half an hour it has been shown (Dornfield^{43,44}) that the contents of the cells are stratified according to their relative densities. For example, in the case of the cortical cell the nucleus is thrown centrifugally together with the mitochondria. The Golgi apparatus is situated above the nucleus (centripetal to it) and is often broken up into small granules (Text-fig. 18). In the medullary cell the nucleus is of different specific gravity relative to the cytoplasm and it is thrown centripetally, although the chromatin which it contains is thrown centrifugally, within the nuclear membrane. The Golgi apparatus is centripetal to the nucleus and the mitochondria are thrown centrifugally (Text-fig. 20). It is obvious that if such an ultracentrifuged adrenal is stained for vitamin C the disposition of the granules within the cell will provide an indication of whether mitochondria and Golgi apparatus contain the vitamin. Dr. J. St. L. Philpot has kindly ultracentrifuged the adrenal gland of a rat for the author for this purpose. The gland was ultracentrifuged at the speed producing a force of 300,000 g. for three-quarters of an hour. Microscopical examination after staining with the acetic acid-silver nitrate reagent showed three types of disposition of the granules. First some cells in both cortex (Text-fig. 23) and medulla (Text-fig. 19 and Plate 3, Fig. 5) showed granules located in both the mitochondrial and Golgi regions; secondly, some showed the granules located principally in the Golgi region (Text-figs. 17 (A and C), 21, 22, 24; Plate 3, Figs. 6, 7, and 8); and thirdly, in others the granules were located principally in the mitochondrial region (Text-figs. 17 B and 25).

The various results which have been quoted suggest very strongly that vitamin C may frequently be associated with the Golgi apparatus of a wide variety of cells.

d. Physical Condition of the Golgi Apparatus

Hirsch states that the evidence available suggests that the Golgi apparatus may exist as a uniformly staining body ('pre-substance') or as a substance showing osmiophilic externum and osmiophobic internum ('Golgi system'). The results obtained from ultracentrifugation (Beams, King, Gatenby, Dornfield, and others) show that in most cells the specific gravity of the Golgi material is less than most of the other cellular constituents. In uterine gland cells the apparatus is of a very fluid consistency. In thyroid cells (Hellbaum⁶⁴) and in spinal ganglion cells (Brown²⁵) the Golgi apparatus retains its form even though displaced from its normal position in the cell; it is obviously relatively rigid in the cells of these organs. Dornfield⁴⁴ has shown that in transplants of centrifuged adrenal cells the displaced Golgi apparatus returns to its

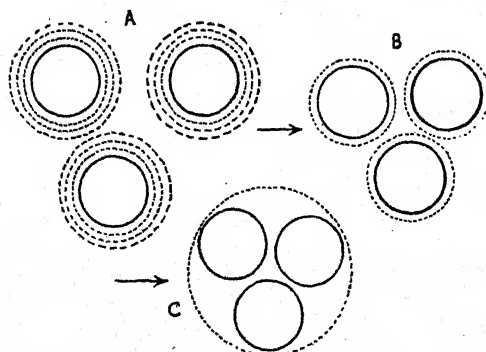
normal position in the cell after a short time and becomes indistinguishable from that of normal adrenal cells.

The apparatus therefore varies in consistency in different cells of the body and probably varies in individual cells according to their physiological condition. The Golgi apparatus in developing male germ and other cells is frequently associated with a differentiated area of cytoplasm near the nucleus which may stain differently from the rest of the cells, called the idiozome.

The interior or 'internum' of the Golgi apparatus seems to be a water-rich area similar to the internal region of the mitochondrion, into which water-soluble substances (e.g. vitamin C) may be passed. The evidence available suggests that the homogeneous bodies (pre-substance of Hirsch) and the osmiophilic portion of the Golgi system are composed of lipo-protein. These homogeneous bodies, may, as Hirsch has suggested, be converted into the Golgi systems by the absorption and concentration into the interior of water and water-soluble substances and by the activity of enzymes which produce the product of the cell within the interior of the homogeneous body. It is possible that the pre-substance may contain a small central core but that it cannot be seen because it is obscured by a thick, heavily impregnated, outer wall. It seems possible that since the 'pre-substance' stains with Janus green B and neutral red it contains proteolytic enzymes. The fact that the Golgi apparatus can hypertrophy, and the possibility that it contains enzymes, suggests that it is able to convert cytoplasm of the cell into extra enzyme material in the same way that some virus particles can convert the tissues of the host into fresh virus material. This implies that the Golgi apparatus can convert the elements of the cytoplasm into material which possesses more free energy than they possessed before. This sort of change, however, is not unusual when we are dealing with living tissues because it is carried out by every organism when it converts food into living material.

Hirsch⁶² has listed points of resemblance between the Golgi apparatus and coacervates. The term 'coacervate' comes from the Latin *acervus*, a crowd or swarm, and it refers to the structures formed during the slow separation of a colloid from solution. Coacervates are formed of a large number of small droplets which lump together. In a typical colloidal solution each particle is surrounded by concentric sheaths of water. The inner layer is very firmly bound to the particle and the successive water layers are less and less strongly bound until there is a gradual gradation into the free water of the sol. One can conceive each colloid particle, therefore, as being surrounded by an envelope of water. Change of electric charge on the colloidal particles, or the addition of dehydrating agents, causes the removal of the envelope of loosely bound water, leaving only the strongly bound water attached to the particle. In this

condition the particles come into contact with one another, and the various water shells join up (Text-fig. 26).



TEXT-FIG. 26. Various stages in the formation of a coacervate. The dotted lines represent watery shells around the colloidal particle.

Hirsch makes the following comparisons between coacervates and Golgi systems.

1. Coacervates are stable systems which are in equilibrium and do no work. On the other hand, Golgi systems do perform work.
2. We know the chemical composition of the various coacervates which have been formed, but we do not know for certain whether the chemical substances of the Golgi system are those which could form coacervates.
3. The form of coacervates and Golgi systems is similar.
4. The state of the Golgi system changes, varying from liquid to solid. It is possible by varying the external conditions to produce similar changes in consistency of the coacervates.
5. Various substances can be adsorbed on the exterior of the Golgi systems. Coacervates also possess this ability.
6. An externum and internum are present in both Golgi systems and coacervate, but the externum is often very irregular in the case of the Golgi system.

We see in this comparison some striking similarities between Golgi systems and coacervates, but probably the greatest argument against such homology is the first quoted comparison, i.e. that the coacervate is a relatively stable system and the Golgi apparatus has free energy and is able to do work. If the Golgi system is a coacervate it is one which is very different from any of those with which we are familiar.

The Golgi apparatus possesses the ability to adsorb various dyes. It seems able to adsorb basic dyes *in vitro* and acid dyes only after injection. A basic dye, neutral red, is claimed by Hirsch to stain only

the 'pre-substance' of the Golgi system. Chlopin³² and others state that the neutral red forms new vacuoles in the cytoplasm. These vacuoles contain protein in solution and can therefore be fixed and stained. Chlopin calls these bodies the 'crinome'. Trypan blue, if it is injected (whereby it may be combined with a protein), becomes located exactly (according to Bowen²³) in the region of the Golgi apparatus of certain cells. Kedrowsky⁸⁰ has also found that if acid dyes are mixed with wheat flour they can be adsorbed into the Golgi material of *Opalina*. This again suggests the attachment of an acid dye to a protein. In fact Hirsch states that the evidence available shows that acid dyes such as trypan blue can only be adsorbed on to the Golgi apparatus if they are combined with an albumen carrier. For an analytical review of vital staining see Ludford, 1933⁹⁷. Keller and Gicklehorn⁵² have found that the majority of basic dyes, in the cell or in protein solutions, bear a negative electrical charge; the adsorption of basic dyes by the 'pre-substance' suggests that the latter bears a positive electrical charge. Hirsch states also that there is evidence that the Golgi system itself is positively charged. The fact that the apparatus in some cells adsorbs vitamin C, which probably carries a negative charge, supports this view. The fact that in the same cell types on different occasions the mitochondria adsorb vitamin C and on others the Golgi apparatus does so may suggest a varying charge on the two structures.

Hirsch and Bretschneider⁶² have found that if iron sugar is fed to an animal the iron is adsorbed on to the Golgi apparatus and not on to the mitochondria or the nucleus (Plate 4, Fig. 1). Kedrowsky⁸⁵⁻⁸⁷, however, found that iron could only be adsorbed by the Golgi bodies of *Opalina* if it was mixed with peptone. It is of interest that a substance known as iron ascorbate (iron salt of vitamin C) exists and may have a physiological function; the fact that iron and vitamin C both aggregate at the Golgi apparatus suggests the sort of function the apparatus may have, namely, that of bringing various reactants into contact with each other.

Van Teel⁶³ has shown that Golgi systems also adsorb compounds of copper and gold. Silver compounds are also localized in this region of the cell, and Kedrowsky⁸⁵ has shown that the Golgi bodies of *Opalina* can adsorb bismutose (a compound of albumen and bismuth) and protargol (a compound of albumen and silver).

e. Function of the Golgi Apparatus

The adsorptive properties of the Golgi apparatus mentioned above lend colour to the speculation that the Golgi apparatus acts as a condensation membrane. Of this theory Kirkman and Severinghaus⁸⁹ state:

'A great deal of work strongly suggests that the Golgi apparatus neither synthesises secretory substances nor is transformed directly into them; but it

acts as a condensation membrane for the concentration, into droplets or granules, of products elaborated elsewhere and diffused into the cytoplasm. These elaborated products may be lipoids, yolk, bile constituents, enzymes, hormones, or almost any other formed substance.'

The conception of the Golgi apparatus purely as a condensation membrane may be only half the truth, for it seems possible that synthetic products may also be formed in the interior of the apparatus. Bowen speaks of the Golgi apparatus as 'a great intracellular center of chemical synthesis or enzyme formation'. Kirkman and Severinghaus lay considerable stress on the condensation membrane hypothesis and point out that since the Golgi apparatus segregates acid dyestuffs into droplets 'one must again conclude that the topographical appearance of secretory droplets, as evinced in the reaction of kidney and liver cells to acid vital dyes, suggests nothing so much as a segregating and condensing function for the Golgi substances'.

Earlier workers believed there was an association between the Golgi apparatus and secretion, and this was put forward more definitely by Nasonov¹¹⁰ in 1923. Bowen's brilliant series of investigations proved conclusively the association of the Golgi apparatus with the production of secretion droplets in various types of exocrine gland. Droplets of secretion appear in the interstices of the Golgi network, and as they grow in number so the apparatus hypertrophies, and finally, when the cell carries its full load of secretion, the apparatus appears to break up and small pieces become attached to some of the secretory granules (Plate 4, Figs. 4 and 7). Bowen worked with the well-known externally secreting glands. There is evidence also that the apparatus is associated with the secretion of bile by the liver, of synovial fluid by the synovial membrane, in the production of the enamel of teeth by ameloblasts⁹ (Plate 4, Fig. 2), the production of pigment by the cells of the iris epithelium, the formation of fatty yolk in eggs and of the acrosome in spermatozoa. Hypertrophy of the apparatus is also found in cells which are undergoing cornification and in connective tissue which is forming fat, and in the formation of cartilage. Cramer and Ludford⁴¹ state that the apparatus is actively concerned in the process of resynthesis of fats from the fatty acids and glycerol absorbed by the intestinal cells.

It has been said that because a cell organ such as the Golgi apparatus hypertrophies in a cell that it is not necessarily bound up with the secretion of certain droplets which are seen near it or in its interstices. It may be true that the actual substance of the Golgi apparatus may not be modified into secretory droplets but the syntheses of the cell take place as a result of the catalytic action of surfaces, and the hypertrophy and partial fragmentation of the Golgi apparatus are undoubtedly the result of the necessity within the cell for an increase of synthetic surface. It is possible, too, that enzymes are adsorbed on to the surface of

the apparatus. The same argument applies to mitochondria. When they are filamentous they are providing the smallest, and when they are in granules they are providing the greatest, possible surface. One reason why the surfaces of a cell are so important, and why they are probably the seat of syntheses, is that surfaces are invariably regions of free energy. Synthesis requires energy, and if the chemical reactions of the cell are to obey the Second Law of Thermodynamics, such syntheses must take place where free energy is available. R. S. Lillie²⁴ has stated that various experiments by Warburg, Harden, McLean, and others have shown that oxidative reactions occur most actively in the living cell in contact with solid or structural cell material, i.e. at the boundary of protoplasmic phases, in other words at the surfaces of membranes, of fibrils, granules, and other solid cell structures, which appear to have a catalytic or accelerating influence on these reactions. This has been tested with living cells by the use of reagents which become coloured on oxidation. Lillie says that in areas of the cell surrounded by membranes a 'variety of physiological or metabolic activities may proceed side by side in the same cell without interfering with one another'. Lillie quotes the Golgi apparatus and the mitochondria as two examples of such areas. It may be significant that these organelles are often associated with other cell membranes, e.g. the mitochondria are often aggregated near the nucleus or the cell membrane and the Golgi apparatus is almost invariably associated with the nuclear membrane.

It is known that the velocity of any reaction taking place at a surface will be greater the larger the surface exposed. In colloid systems there exists what Alexander² calls a zone of maximum colloidalness, which means that in such a system too great a degree of aggregation results in only a relatively small surface area being available and a given reaction proceeds too slowly. In a condition of too great a dispersal the reaction proceeds too rapidly. Therefore to obtain the appropriate speed there must be a balance between the two states of dispersal. Once again we may apply this analogy to the mitochondria and the Golgi apparatus. There may be a mitochondrial-Golgi surface to cell volume ratio for which the cell has its optimum function. An alteration of this surface: volume ratio by bacterial toxins, undue amounts of hormones or drugs, or deficiency of minerals or vitamins may be one of the factors involved in the state known as ill health. Examples are the formation of large mitochondrial masses in scurvy (Bourne¹⁷) and the increase in the amount of Golgi material in the nerve cells in beri-beri (Chang³¹). Also Horning has investigated the effects of acute and chronic morphine poisoning in the Golgi apparatus of various tissues of the white rat. In the spinal neurones of animals with acute morphine poisoning (as a result of massive injections of morphine) he found that there was a hypertrophy and fragmentation of the Golgi material. These changes

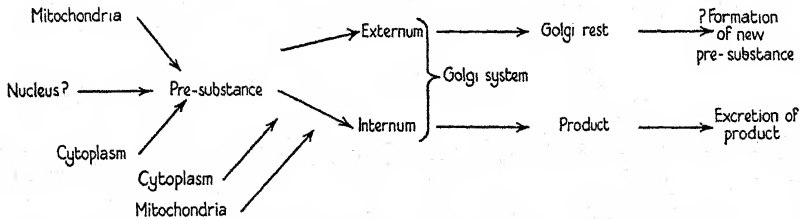
are beautifully illustrated in Plates 5 and 6, the photomicrographs for which were kindly supplied by Dr. Horning.

In experimental pneumonia Tonutti¹²⁸ has found that alveolar phagocytes mobilized in the lung contained a large number of vitamin C granules. Since vitamin C is usually segregated in either the Golgi apparatus or in the mitochondria, this suggests that one or the other of these organelles has broken up into innumerable granules and has thus enormously increased the amount of surface available (Plate 1, Fig. 6). It has been shown by Martinson and Fettisenko^{105,106,107} that vitamin C stimulates both lipolysis and proteolysis and therefore such a cell is well equipped to deal with phagocytosed material.

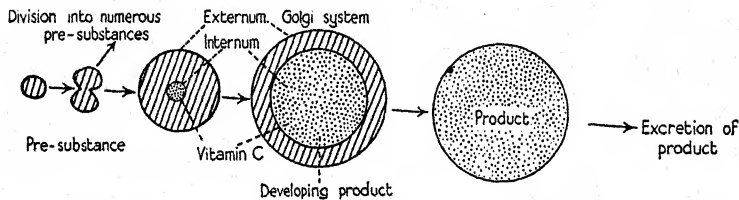
Bayliss⁷ has described the living cell as a heterogeneous system. In a homogeneous system all the molecules are equally accessible to external influences, either chemical or physical, and in heterogeneous systems only those at the surface of each phase are so accessible. Therefore, only the superficial molecules of the mitochondria or Golgi apparatus are capable of direct reaction with the cytoplasm. Although we cannot conceive of mitochondrial or Golgi apparatus activity apart from the cytoplasm in which it lies, we must remember that some substances, e.g. vitamin C, can pass into the interior of these organs, and that therefore reactions can proceed inside both the mitochondria and the Golgi apparatus which would be quite different from those proceeding at the surface. It seems that both Golgi apparatus and mitochondria are in a state of chemical balance and that changes in the permeability of the cell membrane or of their own membranes may swing their activity from rest to synthesis or lysis.

The Golgi apparatus appears to be intimately associated with cellular synthesis, and it has been known for some time that in scurvy synthetic processes of cells are inhibited. Hirsch claims that when a cell is about to form a 'product', the Golgi apparatus adsorbs vitamin C. He says that prior to the formation of secretion droplets in pancreas cells, vitamin C can be demonstrated in the Golgi region. Klein⁹⁰ has shown that osteoblasts may also contain aggregations of vitamin C in the same region of the cell. As mentioned before, fibroblasts *in vitro* show the presence of vitamin C in the scattered Golgi material only when the cell is producing fat. Cartilage cells in the chick embryo prior to the hypertrophy which precedes bone formation show dense aggregations of granules, not only in the Golgi apparatus but packing the rest of the cytoplasm as well. Also, in the histogenesis of other organs in the chick embryo, particularly the liver and the brain, the cells show (as has already been mentioned) an aggregation of vitamin C granules in those regions of the cell where one would normally expect a Golgi apparatus to be situated. In healing wounds (Bourne—unpublished work) cells are present which contain abundant black granules, many of them

aggregated near the nucleus in what appears to be the Golgi region of the cell but others lying in the body of the cytoplasm (these cells are probably macrophages and may be assumed to be producing enzymes for the intracellular digestion of engulfed material). It is noteworthy, too, that the outer cells of the zona fasciculata of the adrenal gland



TEXT-FIG. 27. Relationship of Golgi apparatus to other parts of the cell and to the formation of a product. Redrawn after Hirsch⁸².



TEXT-FIG. 28. Relationship of vitamin C to the Golgi apparatus. Redrawn after Hirsch⁸².

show vitamin C granules aggregated in the Golgi region, whereas this is not always so in the inner cells of this zone. Bennett¹¹ has shown that it is the outer cells of the zona fasciculata which contain the biologically active 17-keto-steroids.

These facts lend strong support to the conception that vitamin C through its association with the Golgi system plays a part in a wide variety of cellular syntheses, but it seems unlikely that the vitamin plays a direct part in the synthesis of such different chemical substances. Tonutti thinks that the Golgi apparatus stores vitamin C and liberates it slowly into the cytoplasm in sufficient amounts to prevent oxidation of the cell products.

A more likely explanation seems (since the metabolism of cells is raised when they are carrying out synthetic processes) that unless these various products are being protected by being produced in, or adsorbed on to, a specially segregated, highly reducing area of the cytoplasm, they would be oxidized as rapidly as they are formed.

Wolf-Heidegger¹³⁸, in a recent communication has suggested a converse relationship between vitamin C and the Golgi apparatus in the cells of the teli of the choroid plexus. He claims that the vitamin

enters the cerebro-spinal fluid from the blood in this region. In the blood the vitamin is chiefly in the reversibly oxidized form and in the cerebro-spinal fluid it is in the reduced condition. During the passage of the vitamin across the cells of the choroid plexus, reduction must occur. Wolf-Heidegger states that the vitamin is adsorbed on to the Golgi apparatus and he believes that this is the region where reduction takes place. If this is so it is possible that the second reducing substance may be glutathione which, incidentally, does not react with acetic acid-silver nitrate. There is a roughly parallel distribution of glutathione and vitamin C in the body and the two substances are known to be capable of forming an oxidation-reduction system. It should be borne in mind that the Golgi apparatus in cells other than those of the choroid plexus may be the locus of a 'redox' system based on vitamin C and glutathione.

One can conceive of the function of the Golgi apparatus in gland cells, but it is difficult to understand its function in non-secretory cells such as the nerve cell. Some workers have claimed that its function in the nerve cells of *Helix* is to secrete fat. A more likely activity is suggested by Brambell²⁴, based on his work on *Helix*. He believes that in the neurones the apparatus is responsible for the secretion of the Nissl granules. He found Nissl granules distributed in the same manner as the Golgi apparatus, and Nissl granules were most marked in those cells in which the apparatus is very active. Andrew⁵ in the Purkinje cells of young and adult mice found distinct Golgi nets, but in senile animals he found the Golgi material in the form of scattered granules.

Cajal^{28a} and Penfield^{111a} found a fragmentation and regression of the Golgi apparatus of spinal neurones following section of their axones.

The Golgi apparatus increases in amount in beri-beri (Plate 4, Figs. 5 and 6). This gives the impression that it is undergoing a compensatory hypertrophy. If this is so it is of special interest because of the chemical changes found in nervous tissue in beri-beri by Peters and his co-workers.¹¹² It is tempting, therefore, to postulate that the Golgi apparatus is associated with carbohydrate metabolism.

In the thyroid gland Cowdry^{39a} has suggested that the reversed polarity of the Golgi apparatus which is seen in some cells is an indication of reversal of secretion; that the cell instead of discharging its products into the lumen of the follicle is discharging them directly into the blood-stream. This reversal of polarity as an indication of reversal of secretion is not upheld by all other workers. For a discussion of the Golgi apparatus and thyroid secretion see Chapter VIII.

Courrier and Reiss³⁶ stated that the cells of the parathyroid gland were arranged in cylindrical columns, that these columns were drained by a central capillary and supplied with peripheral nutritive capillaries. The authors claimed that the Golgi apparatus of the parathyroid cells

was orientated between the cell nucleus and the central vein. Other workers, however, have found that many parathyroid cells do not show this orientation of the Golgi apparatus.

Reiss¹¹⁵ stated that in the hypophysis of the cat the chromophobe cells showed no particular orientation of the apparatus, suggesting that these cells were in the resting condition. In the acidophil cells the Golgi apparatus occurred between the nucleus and the central area. In basophils it was placed peripherally. Reiss stated that all stages of transition between the two types of cells could be observed, and he expressed his belief that the cell can pour out a secretion at the periphery and then reverse and discharge a different substance into the centre of the cluster. Severinghaus¹²³ found, however, that the Golgi apparatus was not orientated towards the lumen of the blood capillaries in either basophils or acidophils. He claims that there is no justification for using its position in the cell as an indication of secretory activity. Severinghaus also found two distinct types of Golgi apparatus in the chromophobes. One type of apparatus was comparable with that found in acidophils and the other with that found in basophils. He believes that this is evidence that these cells arise separately from the chromophobes and that they are not mutually interchangeable.

In view of the results obtained by Bowen²³ and others on the role of the Golgi apparatus in the production of secretory droplets in externally secreting glands, an attempt was made by Bourne¹⁶ to demonstrate that the Golgi apparatus of the adrenal was associated with the production of the lipid droplets of the cortical cells. Such an association would be of special interest because Bennett¹¹ has shown that at least in the outer half of the zona fasciculata the lipid droplets contain biologically active 17-keto-steroids. Clear-cut results similar to those of Bowen for externally secreting glands could not be obtained. It was found, however, that in cells containing few or no lipid droplets in the secretory part of the cortex the Golgi apparatus was invariably small and compact. On the other hand, cells which were full of lipid droplets could be divided into two types, those with a small apparatus, and those with a hypertrophied and partly fragmented apparatus. It appeared that at some stage the Golgi apparatus might play a part in the maturation of the lipid droplets. Types of Golgi apparatus present in the adrenal cortex and medulla can be seen in Plate 3, Figs. 1A to H. These may be compared with the vitamin C preparations of adrenal cells, Plate 3, Figs. 2, 3, and 4.

f. Summary of the Golgi Apparatus

The Golgi apparatus is a cell organelle of such variable shape in fixed preparations that it is almost certain that in the living cell it is constantly, but extremely slowly, changing its form.

It very likely contains enzymes or can synthesize them from the cytoplasm or adsorbs them on to its surface.

Without doubt the Golgi apparatus acts as an important condensation membrane, adsorbing dyes, metals, and organic substances such as vitamin C. These substances may pass through into the Golgi internum and some of them may play a part in the formation of the synthetic product of the cell.

The Golgi apparatus may exist without an 'internum', in which condition it is described by Hirsch as 'pre-substance'.

The Golgi apparatus is certainly involved in the production of the secretion of exocrine glands and it may also be associated with the formation of endocrine products.

It appears to play some part in the differentiation of embryonic cells, for it is active during histogenesis.

Vitamin C is present in the Golgi apparatus of a wide variety of differentiating embryonic cells, and it appears to be concentrated in the Golgi apparatus of cells which are engaged in active synthesis of various substances. From these facts it appears that its presence may be due to the fact that it is necessary for these products to be formed in, or adsorbed on to, a specially segregated highly reducing area of the cytoplasm in order to prevent them from being oxidized in the increased cell metabolism.

The Golgi apparatus appears to be involved in the passage of vitamin C across the cells of the placenta and kidney. By the use of the Golgi apparatus as a segregation apparatus the cell prevents the serious changes in rH which would occur as a result of the diffusion across it of a powerful reducing substance such as vitamin C.

The Golgi apparatus appears to be composed basically of a lipoprotein.

PLATE I

FIG. 1. Myoblast from culture of ventricle of 6-day chick embryo. The mitochondria may be seen as rods and long filaments. From Richardson (1934).

FIG. 2. Telophase in myoblast from culture of ventricle of 6-day chick embryo. The mitochondria are distributed more or less equally between the two daughter cells. From Richardson (1934).

FIG. 3. Section of *Opalina*. The mitochondria (Mt) may be seen as small rods associated with grey spheres, the vegetative granules (Vg). From Richardson and Horning (1931).

FIG. 4. Vitamin C preparation of cell from adrenal medulla of rat. The granules have a mitochondrial appearance. (Retouched.) Compare with Fig. 5. $\times 1,200$.

FIG. 5. Mitochondrial preparation of cell from adrenal medulla of the guinea-pig. From Mulon, after Rogoff and Stewart (1932).

FIG. 6. Histiocyte from infected lung. Stained to demonstrate vitamin C. The entire cell may be seen to be packed with granules. From Tonutti (1938).

FIG. 7. Vitamin C preparation of corpus luteum cells showing mitochondrial appearance of granules. After Giroud (1938).

FIG. 8. Vitamin C preparation of anterior pituitary gland of rat showing mitochondrial appearance of granules in chromophilic cells. The chromophobe cells give no reaction with the silver nitrate reagent. (Retouched.)

PLATE II

FIG. 1. Living fibroblast from culture of rat heart. Dark ground illumination. The nucleus may be seen as a black oval mass near left border of light area represented by the cell. Immediately to the right of the nucleus and in contact with it is an irregular black area speckled with white. This is the Golgi apparatus. From Ludford (1935).

FIG. 2. Electron microscope photograph of an apparently homogeneous silver granule in a photographic emulsion. (Magnification 25,000 diameters. By permission of Science Service, Washington, and the Research Laboratories of Kodaks, Ltd.)

FIG. 3. Epithelial cell from culture of chick liver. The Golgi apparatus may be seen to the right of the nucleus. The division into inner and outer portions may easily be seen. From Richardson (1934).

FIG. 4. Hepatic cell of mouse. Kolatchev preparation. The Golgi apparatus may be seen surrounding the nucleus. (Retouched.) From Tarao (1939).

FIG. 5. Hepatic cells of mouse digested by pepsin and stained with Nile blue sulphate. Two nuclei are visible, and the stained material may be seen to occupy a similar position to the Golgi apparatus demonstrated by Kolatchev's method in Fig. 4. (Retouched.) From Tarao (1939).

FIG. 6. Golgi preparation of cells from secretory zone of adrenal cortex of cat. From Bennett (1940).

FIG. 7. Golgi preparations of zona glomerulosa cells of adrenal of cat. From Bennett (1940).

FIG. 8. Stages in secretion of zymogen granules in the pancreas. For details see text. The second cell shows the passage of small droplets from the mitochondria to the Golgi field. From Hirsch (1939).

FIG. 9. The staining of the egg of *Aplysia* by various techniques. The Speicher granula may represent the Golgi material. It may be seen that these granules react with neutral red and appear to contain vitamin C. From Ries (1937).

FIG. 10. Kidney cells of rat stained with trypan blue and vitamin C reagent. Black granules can be seen surrounded by a grey envelope which in the actual preparations is stained blue by the trypan blue. From Hirsch, after Tonutti (1939).

FIG. 11. Vitamin C preparation of human decidua cell showing localization of vitamin C in the Golgi region. From Giroud (1938), after Tonutti and Plate.

PLATE III

FIG. 1. *a, b, c, d, e, f, g.* Golgi apparatus in adrenal cortical cells of marsupials. *h.* Golgi apparatus in medullary cell of opossum adrenal. (Retouched.) From Bourne (1934). *a* and *b* $\times 2,500$; *c, d, e, f* and *g* $\times 1,200$; *h* $\times 1,800$.

FIGS. 2 and 3. Vitamin C preparations of cortical cells of rat adrenal (Retouched.) $\times 2,500$.

FIG. 4. Vitamin C preparation of medullary cell of rat adrenal (retouched). Compare with Fig. 1 *h.* $\times 2,900$.

FIGS. 5 and 6. Medullary cells from ultracentrifuged rat adrenal stained to demonstrate the distribution of vitamin C. Most of the granules can be seen to

occupy the position usually taken up by the Golgi apparatus in centrifuged adrenal medullary cells. In Fig. 5 some vitamin C granules are in the same region as the mitochondria. The chromatin of the nuclei is centrifuged to one end of the nucleus. (Retouched.) Fig. 5 $\times 2,500$; Fig. 6 $\times 1,200$.

Figs. 7 and 8. Cortical cells from the ultracentrifuged rat adrenal, stained to demonstrate vitamin C. The vitamin granules are in the position usually taken up by the Golgi apparatus in ultracentrifuged cortical cells. (Retouched.) $\times 1,800$.

PLATE IV

FIG. 1. Intestinal cells of *Ascaris*. First two cells 20 minutes after feeding with iron sugar, second two cells 30 minutes after feeding with iron sugar. The first and third cells are stained to demonstrate the presence of iron. The second and fourth cells are stained by an osmium technique to demonstrate the Golgi apparatus. The correspondence between these two preparations shows that the Golgi apparatus plays an important part in the absorption of iron. From Hirsch (1939).

FIG. 2. Golgi apparatus in ameloblasts. As the cells commence to produce enamel the Golgi apparatus may be seen to reverse its position in the cell. From Hirsch (1939), after Beams and King.

FIG. 3. Regression of the Golgi apparatus in a neurone after section of its axone. From Hirsch (1939), after Cajal.

FIG. 4. Association of Golgi apparatus with secretion droplets in the sub-maxillary gland of the cat. *n* = nucleus. From Bowen (1926).

FIGS. 5 and 6. Showing hypertrophy of Golgi apparatus in neurones in beri-beri fowls. Fig. 5 a normal cell; Fig. 6 cell from beri-beri fowl. (Retouched.) After Chang (1932).

FIG. 7. Association of Golgi apparatus with secretory droplets in parotid gland of cat. *n* = nucleus. From Bowen (1926).

PLATE V

FIG. 1. Typical morphology of the Golgi apparatus in the neurone of a healthy normal albino rat. Note the thinness of the anastomosing strands of the apparatus.

FIG. 2. Appearance of the Golgi apparatus in the neurone of a white rat killed 3 hours after a massive injection of morphine.

FIG. 3. Polymorphic structure of the Golgi apparatus in the neurone of a white rat killed 6 hours after a massive injection of morphine. The reticulum has completely disintegrated, giving rise to incomplete ring-like structures.

FIG. 4. Neurone of rat at same stage as in Fig. 3. Ring-like formations of the Golgi apparatus also present.

The photomicrographs for this plate were kindly supplied by Dr. E. S. Horning.

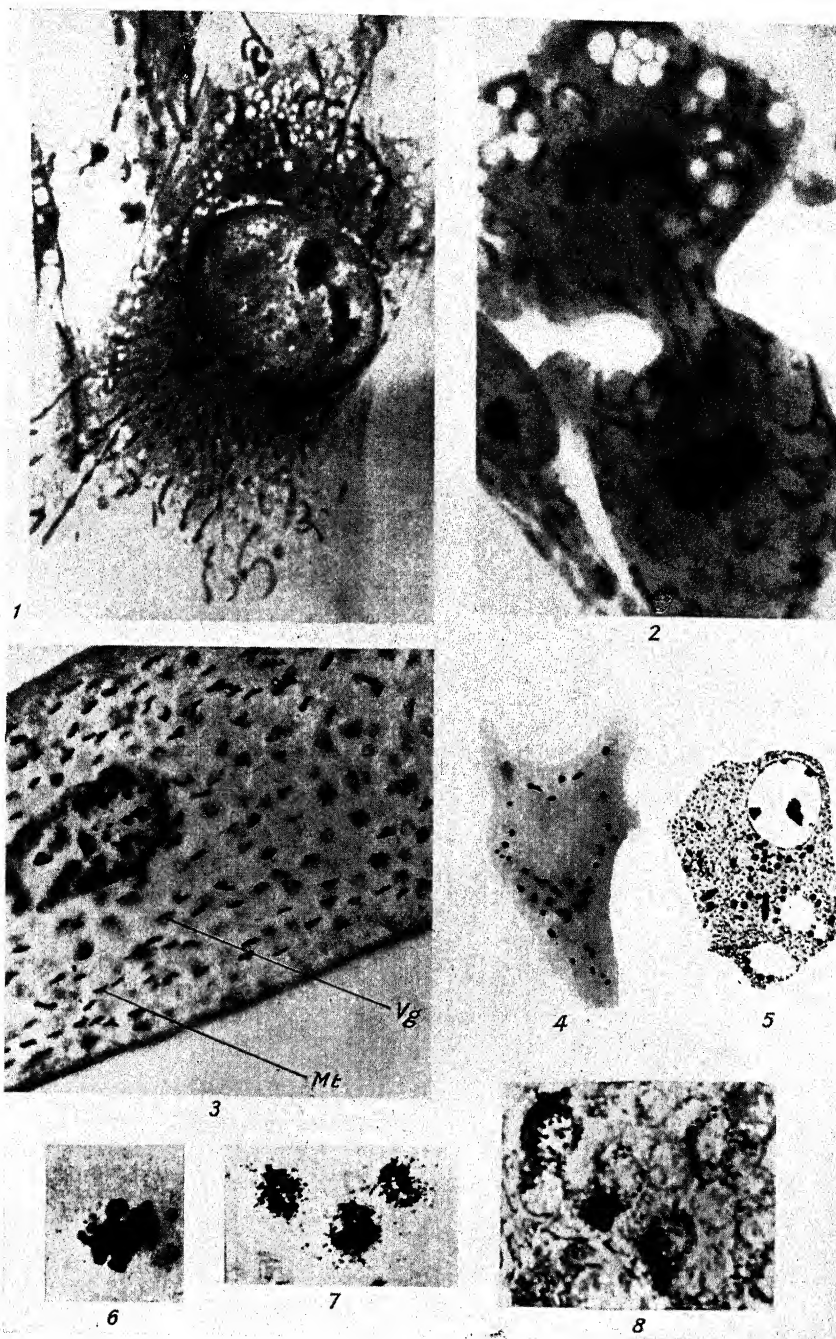
PLATE VI

FIG. 1. Neurone of white rat killed 7 hours after a massive injection of morphine. The Golgi apparatus is hypertrophied and fragmented.

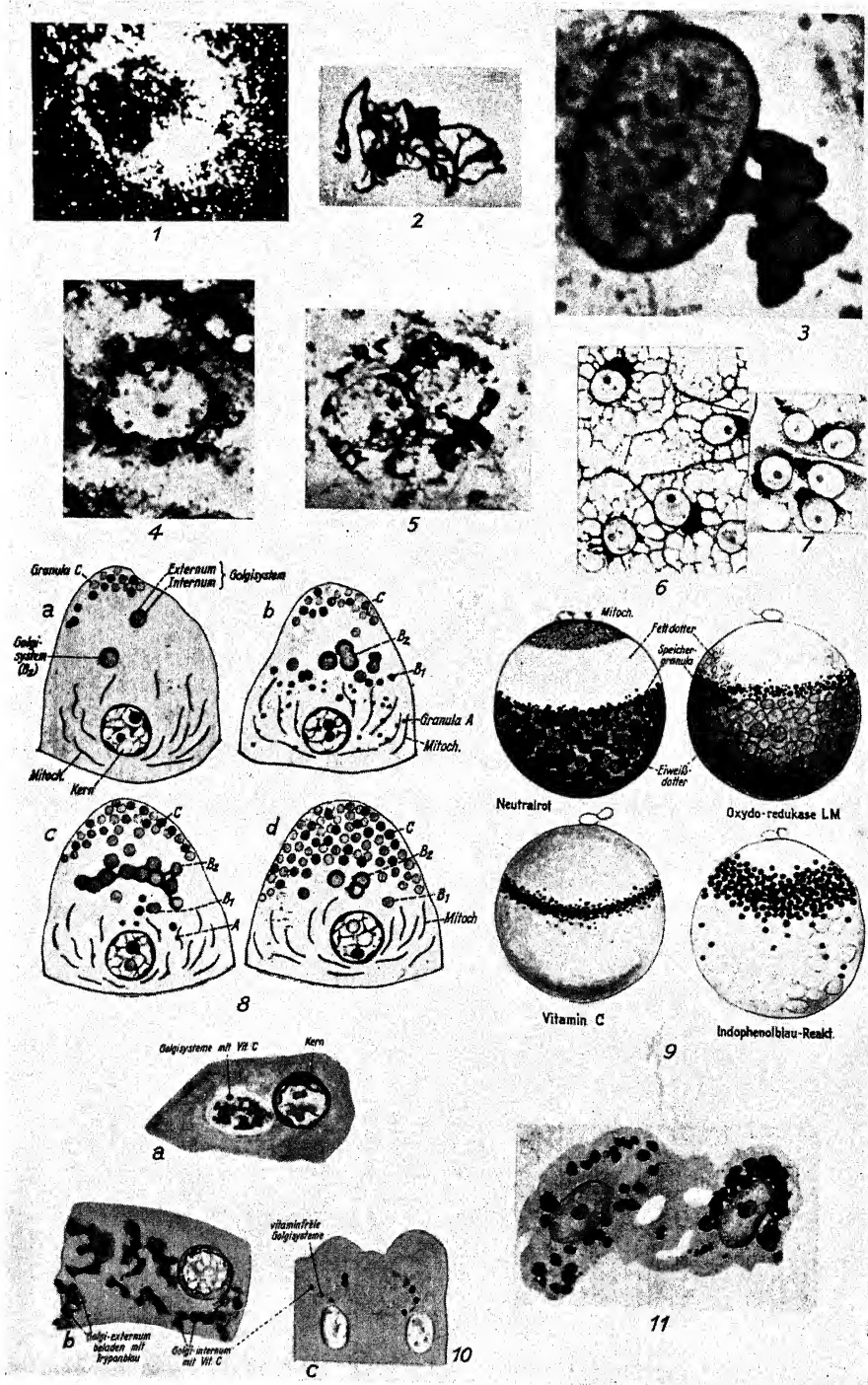
FIG. 2. Neurone of white rat killed 8 hours after a massive injection of morphine. The Golgi reticulum is disintegrated through the body of the cell.

FIGS. 3 and 4. Neurones of white rat after massive injection of morphine, showing morphological variations of the Golgi apparatus.

The photomicrographs for this plate were kindly supplied by Dr. E. S. Horning.



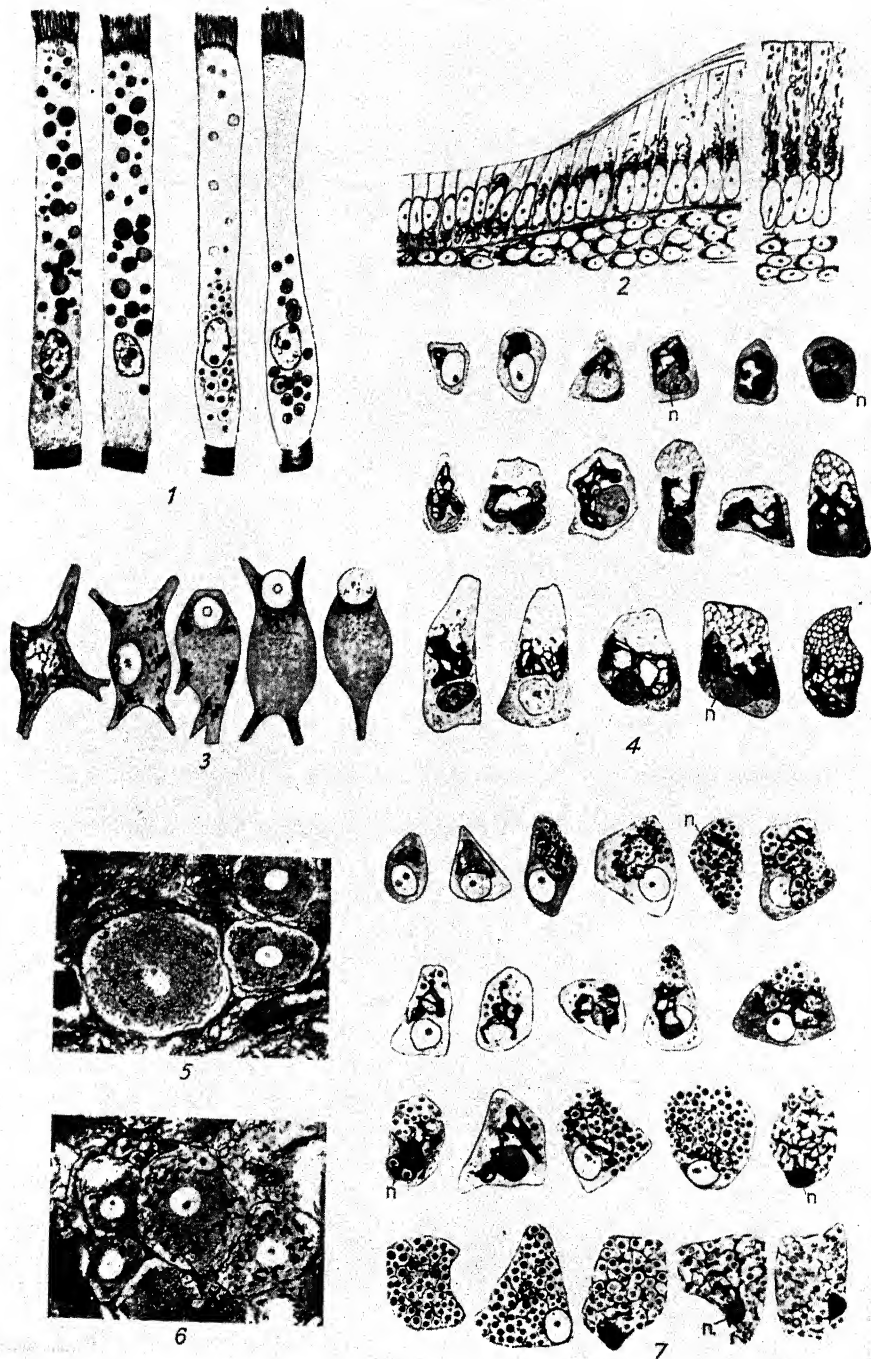
Mitochondria and vitamin C in various cells.



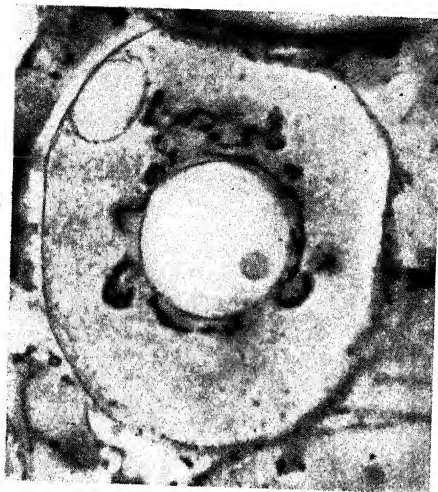
Golgi apparatus and vitamin C in various cells.



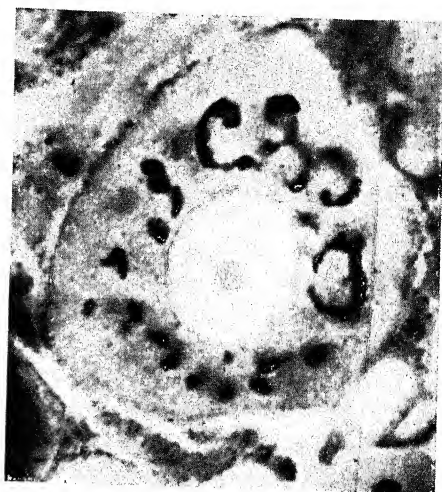
Golgi apparatus and Vitamin C in adrenal cells.



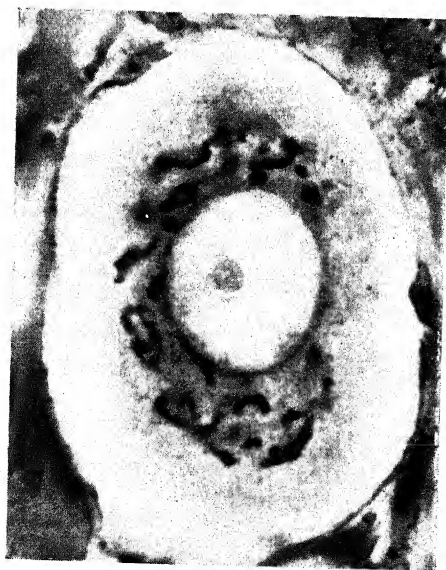
Golgi apparatus in various cells.



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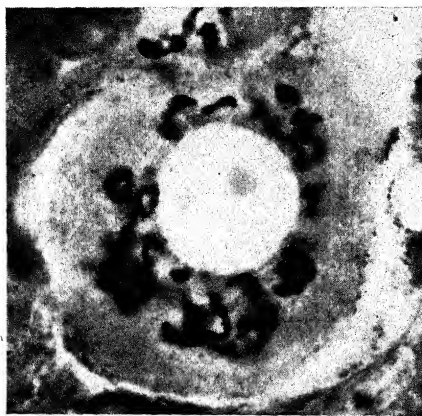
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G. Bourne

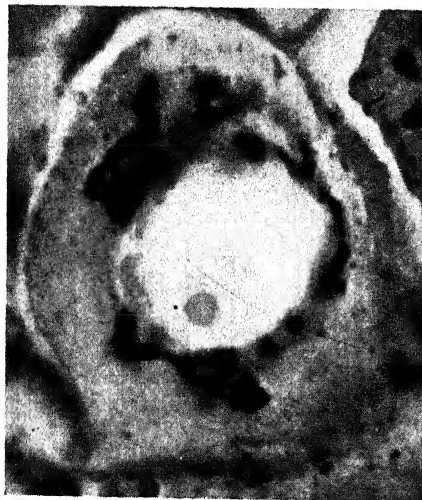
Golgi apparatus in morphine poisoning.



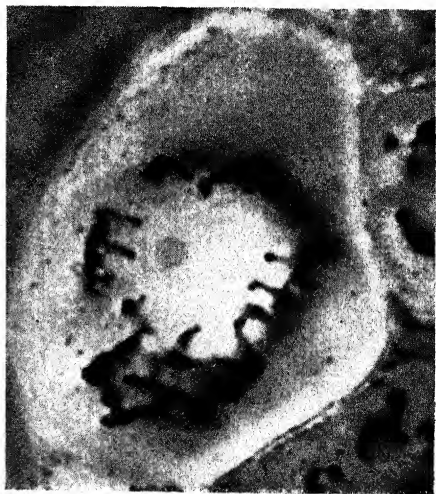
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4

Golgi apparatus in morphine poisoning.

CHAPTER V

NUCLEUS, CHROMOSOMES, AND GENES

By M. J. D. WHITE

i. INTRODUCTION

WHEREAS the general physiology of the cytoplasm has been investigated by many of the usual physiological techniques, the study of the nucleus has been advanced by more specialized methods of a type unknown in ordinary physiological laboratories. The chief of these methods have been anatomical studies of the nuclear cycle, supplemented by chemical and genetical investigations of the chromosomes. Modern cytogenetics is thus a composite science which utilizes very diverse techniques. To understand the problem of chromosome structure and to have a clear mental picture, both of the extent of our knowledge and of its deficiencies, it is necessary to know something of protein chemistry, the geometry of spirals, the properties of X-rays, and the genetics of *Drosophila*. It is thus hardly surprising that the very great advances made by cytogenetics in the last decade have remained largely unknown to biologists working in other fields. But it is particularly regrettable that they have been very generally unknown in one field (cancer research) where they are directly relevant.

Of all the constituents of the cell the chromosomes are unique in their highly specialized methods of reproduction and in the specificity of their parts. Thus an analysis of the molecular structure of chromosomes is clearly one of the central problems of biology at the present time. It is a prerequisite to any real understanding of the process of mutation and consequently of the physical basis of evolution.

ii. COMPOSITION OF CHROMOSOMES

a. Centromeres and chromosome-ends.

A chromosome is made up of three kinds of structures. First of all there is the 'genetical' part—a sequence of genes arranged in linear order. Situated at some point in this sequence is the *centromere* or spindle attachment, a minute region which seems to organize the formation of the spindle at mitosis and which controls the separation of the daughter chromosomes at anaphase. The centromere divides the chromosome into two arms, which may be equal or unequal in length. The ends of these arms seem to possess special properties which will be explained when we come to consider the problem of chromosome breakage by X-rays; for the present it is merely necessary to state that the

ends have been called *telomeres* by some modern workers in this field. Thus all ordinary chromosomes consist of two telomeres, a centromere, and a sequence of genes. The only chromosomes which are definitely known to possess more than one centromere are those of the germ-line in *Ascaris megalocephala* (White⁵⁴) which are destined to break up into a number of smaller chromosomes in the somatic cells. While certain properties of the chromosomes (such as their power of reproduction) are analogous to the behaviour of virus-particles, others (such as the specific pairing attractions which they exhibit at meiosis and in the somatic tissues of the *Diptera*) seem to be unparalleled in other living systems and have not yet received an adequate physical explanation.

b. Nucleic Acids and Nucleotides

So far as we know at present, there are only two kinds of chemical compounds present in the chromosomes, proteins and nucleic acids. These correspond to some extent to the 'linin' and 'chromatin' of the earlier histologists. They appear to be chemically combined in the chromosomes which are consequently made up of salt-like 'nucleo-proteins'. Although there are a number of different types of nucleic acids it seems unlikely that more than one (or at most two) of these actually occur in the chromosomes. It is thus probable that the specificity of gene action resides in the protein part of the molecule, which retains its continuity throughout the whole of the nuclear cycle, unlike the nucleic acid which waxes and wanes in amount throughout the various stages of mitosis. The fact that many enzymes are now known to be nucleo-proteins suggests that the activities of the genes are also enzymatic in nature.

Nucleic acids are polymers of nucleotides, each nucleotide being composed of one molecule of phosphoric acid, a pentose-sugar, and a purine or pyrimidine ring. The various kinds of nucleotides have different purines or pyrimidines. Apart from this there are two main types of nucleotides, which differ in the nature of the pentose. In one type the sugar is *d*-ribose, while in the other it is desoxyribose. It has been shown that nucleotides of the second type can polymerize to a much greater extent than those of the first. Signer, Caspersson, and Hammarsten⁴⁷ prepared the sodium salt of the desoxyribose nucleic acid and studied its physical properties. They concluded that the molecules were long thin rods the length of which was about 300 times the breadth. The molecular weight as determined by the ultracentrifuge was found to lie between 500,000 and 1,000,000. Since the average weight of a sodium nucleotide is about 330, it seems likely that the polymerized nucleic acid molecules in the chromosomes each consist of about 2,000 nucleotides. These conclusions have been confirmed by the X-ray investigations of Astbury and Bell^{1a} who showed that Wrinch⁵⁵ was

wrong in assuming that the nucleic acid molecules lie at right angles to the protein framework. In reality they lie parallel to the protein fibres, being probably combined with them in a 'zipper-like' manner.

The ordinary reactions of the nucleic acids when exposed to stains such as the aniline dyes depend on the fact that the nucleic acid molecule, being ampholytic, contains basic groups. All types of nucleic acid thus stain with dyes such as rosaniline, crystal violet, and haematoxylin. The Feulgen reaction (staining with Fuchsin sulphurous acid after a preliminary hydrolysis) depends on an entirely different principle—namely, the Schiff reaction for aldehydes, which is given by the desoxy- but not by the ribose-sugars. The Feulgen reaction is consequently not given by all nucleic acids, but only by the desoxy-acids (it may possibly be given by ribose nucleic acid after a particularly prolonged hydrolysis).

A much more satisfactory method for demonstrating the presence of nucleic acids in the cell is by means of ultraviolet absorption spectroscopy. This method, which has been developed by Caspersson, Hammarsten, and Schultz, depends on a specific absorption at about 2,600 Å; it renders possible the detection of exceedingly minute quantities of the acid. Since the absorption depends on the pyrimidine part of the molecule, the method is independent of the type of sugar in the molecule, and in fact gives no information as to whether a desoxy- or a ribose-sugar is present. Using this method Caspersson and Schultz have demonstrated the presence of large quantities of nucleic acids, not only in the chromosomes, but also in the cytoplasm of cells which are about to undergo a series of divisions in rapid succession. Egg cells, meristematic cells in root-tips of plants, and imaginal disk cells in insects were all shown to contain cytoplasmic nucleotides. On the other hand, 'mature' cells which would not have undergone any further divisions were found to possess little or no nucleic acid in their cytoplasm. Whether an accumulation of nucleotides in the cytoplasm actually causes the onset of mitosis cannot be determined at present.

The cytoplasmic nucleotides are probably the reason why so many embryonic cells have a strongly basophilic cytoplasm. In many fixed cells the nucleotides seem to be especially concentrated round the nuclear membrane so as to give rise in stained preparations to a basophilic 'halo' round the nucleus. On the other hand, the spectroscopic observations mentioned above have shown that the nuclear fluid is relatively free from nucleic acids.

There is no evidence that the cytoplasmic nucleotides are polymerized into long chains as those of the chromosomes are, and in view of the fact that they are not stained by the Feulgen reaction it is probable that they are ribose nucleotides. Since very large quantities of nucleic acids are localized on the chromosomes during cell division, it seems

likely that the cytoplasmic nucleotides serve as a source of nucleic acid from which the chromosomes derive their supply. If this is so, the preliminary stages of mitosis must involve the transference of nucleotides from the cytoplasm to the chromosomes, their conversion from ribose to deoxyribose nucleotides, and their polymerization into long chains. Thus we may postulate the existence of a regular cycle of changes which must be reversed at the end of each mitosis when the nucleotides are surrendered by the chromosomes. It is probably significant that the amount of nucleic acid in the chromosomes undergoes a sudden increase at prometaphase, when the nuclear membrane breaks down and substances from the cytoplasm have free access to the chromosomes. Exactly what part nucleoli play in this cycle of transference is not quite clear. These bodies are now known to be connected with particular regions of certain chromosomes, and are hence best regarded as parts of those chromosomes rather than as independent cell organs. It is known that they contain ribose nucleotides and they may represent temporary storage places for nucleic acid.

Usually the transference of nucleic acid to and from the chromosomes cannot be directly observed, but in some special instances the chromosomes, after they have divided, appear to slough off a certain amount of nucleic acid which then forms a definite body lying midway between the two daughter chromosomes as they pass to the poles. This phenomenon is exhibited during the meiotic divisions in the eggs of certain moths (Seiler^{45,46}). A similar process takes place in the meiotic and cleavage divisions of the mite *Pediculopsis* (Cooper¹⁵). In both cases the bodies which are left in the equatorial region of the spindle are Feulgen-negative, but stain with ordinary basic stains such as haematoxylin and the aniline dyes (Plate 1, Figs. 1 and 2). They are thus probably composed of some kind of nucleic acid which does not contain a desoxy-sugar. In *Pediculopsis*, where the chromosome number is only three in the haploid egg, it can be seen very clearly that each of the 'elimination bodies' corresponds to a pair of daughter chromosomes. The 'elimination bodies', both in moths and in *Pediculopsis*, are temporary structures which disappear after each division. It is thus probable that they lack a permanent protein framework such as the chromosomes possess, being mere accumulations of some Feulgen-negative nucleic acid. As Cooper rightly points out, it is not possible to determine what is responsible for the appearance of these elimination bodies in certain divisions but not in others. It is, of course, conceivable that their presence indicates the existence of a second kind of nucleic acid in the chromosomes in the particular instances in which they occur; but it would seem more likely that they are derived from the ordinary desoxy-acid of the chromosomes, a chemical change in the sugar molecule having taken place at the time of extrusion. It is interesting to

note that in the related mite *Pediculoides* no elimination bodies are formed (Patau^{41,42}).

It has been shown by Astbury and Bell^{1a} that the distance between the nucleotides in the long polymerized nucleic acid molecules is of the same order of magnitude (3.3 Å) as that between the successive amino-acid residues in a fully extended polypeptide chain. This apparently accidental correspondence between the spacing of the essential units in these two types of molecule may, in fact, be the key to the true role of the nucleic acids, not only in the chromosomes, but also in viruses and in many enzymatic systems. It has been suggested that the auto-reproductive power of the proteins is in some way associated with their power to polymerize nucleic acids and combine with them.

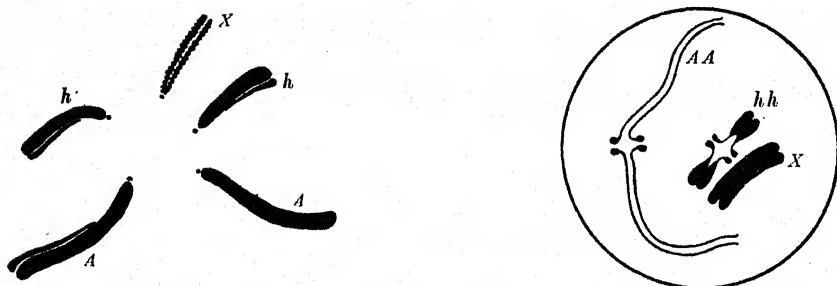
We may thus regard the protein part of the chromosome as a framework which is responsible for the synthesis of nucleic acid. It has long been known that at certain stages the chromosomes appear as long threads with granules or chromomeres distributed along their length. The nucleic acid synthesis is thus confined to certain short lengths of the protein framework where the chromomeres are formed—or is at any rate more highly developed in these regions. In between the chromomeres are 'internodes' in which no nucleic acid (or very little) accumulates. In the giant nuclei of the Diptera salivary glands (*vide infra*) the chromomeres form bands or cross striations. In these salivary chromosomes it is known that each band corresponds to a single gene. In ordinary mitotic and meiotic chromosomes the chromomeres are difficult to study on account of their minute size (near or below the limit of resolution of the microscope) and because adjacent ones tend to clump together, thus diminishing their apparent number.

c. *Heteropycnosis*

Ever since the beginning of the century it has been recognized that some whole chromosomes, or portions of chromosomes, synthesize more or less nucleic acid than the rest of the chromosome set at particular stages of the nuclear cycle. This property of *heteropycnosis* is more especially characteristic of sex chromosomes, but it is also shown by many autosomal regions. It is, in fact, probable that all chromosomes contain segments (either terminal or interstitial) which are heteropycnotic. If these segments are numerous or extensive the chromosomes may appear to be entirely heteropycnotic, while if they are short and few they may pass unnoticed. In many species of grasshoppers there is one autosome in which the heteropycnotic regions are very highly developed, so that the chromosome has been distinguished by various special names (the 'precocious' or 'megameric' chromosome—see Carlson¹¹). *Drosophila*-workers usually speak of *heterochromatic* and *euchromatic*, a terminology which is frequently useful. The earlier investigators were unable to establish any clear distinction between

chromomeres and heterochromatic regions—a difficulty which still persists to some extent.

Two kinds of heteropycnosis exist which are opposite in nature. In *negative heteropycnosis* the chromosome or region in question synthesizes less nucleic acid than the remaining parts of the chromosome set. It thus appears thinner and stains more faintly; usually such chromosomes appear to have a flocculent, 'woolly', outline. In *positive heteropycnosis* more nucleic acid is synthesized, so that the chromosome appears thicker and stains more darkly than the rest. Heteropycnosis may thus be described as a 'natural experiment', the euchromatic



TEXT-FIG. 1. Diagrams of a spermatogonial metaphase and the diakinesis stage of meiosis in a short-horned grasshopper such as *Mecostethus grossus*. Only two pairs of autosomes and the X-chromosome are shown (there would actually be eleven pairs of autosomes). *A* and *A* are a normal pair of autosomes, euchromatic except for a short region next to the centromere. *h* and *h* are a pair of autosomes with an extensive heterochromatic region and a shorter one at the other end of the chromosome. It will be seen that the heteropycnosis of the X is negative in the spermatogonial metaphase, positive at diakinesis. On the other hand, the heteropycnosis of the heterochromatic autosomal regions is only shown at diakinesis, when it is positive in character. (After White⁵⁴.)

regions in the same nucleus constituting a 'control' with which the heteropycnotic ones can be compared.

In some organisms only one of the two types of heteropycnosis exists. Thus in the long-horned grasshoppers (Tettigonidae) the X-chromosomes are positively heteropycnotic at various stages of meiosis, but neither they nor any other chromosomal region ever show negative heteropycnosis. In the short-horned grasshoppers (Acrididae), on the other hand, both positive and negative heteropycnosis exists, and the X-chromosome passes through a regular cycle during the course of spermatogenesis, being negatively heteropycnotic during the early spermatogonial divisions, non-heteropycnotic in the later ones, and positively heteropycnotic during the prophase of meiosis (White⁵⁴). This 'reversal of heteropycnosis' is clearly a fact of considerable interest, but it is not yet known what causes it. Either the protein framework of the X undergoes a change in the course of spermatogenesis, or the composition of the nuclear sap alters in some unknown way. It is of interest that in these short-horned grasshoppers the heterochromatic

regions of the autosomes (which are quite extensive in many species) do not show this reversal, being non-heteropycnotic during all the spermatogonial divisions and positively heteropycnotic in the prophase of meiosis (Text-fig. 1). If, as we have tacitly assumed, heteropycnosis is a property of the protein framework of the chromosome, then there must be three different kinds of framework in the chromosomes of these grasshoppers, one in the X, one in the euchromatic autosomal regions, and one in the heteropycnotic regions of the autosomes.

There is no evidence that the nucleic acid present in either type of heteropycnosis is in any way chemically different from that found in euchromatic regions. Both stain with the Feulgen technique and it is probable that they are identical. Differences in the intensity of staining reactions are possibly to be accounted for by the differences in the ratio amount of nucleic acid: amount of protein.

The early geneticists soon discovered that an association existed (in *Drosophila melanogaster*, at any rate) between heteropycnosis and genetical 'inertness'. Thus the Y-chromosome, which is almost completely 'inert', is positively heteropycnotic at many stages of the nuclear cycle. The proximal end of the X is also heteropycnotic and inert, and so are short regions in the middle of chromosomes 'II' and 'III'. It now seems probable that this association is a very general one which possibly occurs in all groups of organisms—a fact which is of considerable use to the geneticist, since it means that he can predict the genetically active and inert regions of the chromosome set from a rapid cytological examination of his material instead of having to wait for the results of a complete genetical analysis. We may predict, for example, that the 'megameric' chromosome in the short-horned grasshoppers will be mainly inert. It is known, however, that apparently completely heteropycnotic regions (such as the proximal segment of the X in *Drosophila* and the 'differential' region of the human X-chromosome) do contain a few active genes such as *bobbed*, and the genes for haemophilia and colour-blindness in man. It may be that in these instances the genes in question really lie in intercalary euchromatic segments which are too short to be detected under the microscope.

The whole problem of genetical inertness is one of considerable complexity. It was formerly assumed that inert regions contained numerous genes which had somehow lost their function and their power to mutate. More recently Muller and his co-workers have put forward the view that an inert region is one which contains far fewer genes per unit length than an 'active' region. It will be apparent that the difference between these two interpretations is bound up with the question of whether 'internodes' exist between the genes, or whether the latter are really continuous along the length of the chromosome. One thing that

does appear certain is that all the inert regions in *Drosophila melanogaster* are in some way 'homologous', since in the salivary gland nuclei they are all paired together to form a body known as the chromocentre (*vide infra*). It is probable that if inert regions are to be looked upon as built up of 'genes' at all they consist of repeated duplications of the same functionless gene or genes.

It has been known for a considerable time that irradiation by X-rays can cause both mutations of single genes and chromosome breakage. Since mutation only occurs very rarely in the inert regions of *Drosophila* chromosomes it might be expected that chromosome breakage would be correspondingly rare. Kaufmann and Demerec²³ have shown, however, that when *Drosophila* sperm is irradiated, the Y-chromosome undergoes fragmentation about as often as would be expected from a comparison of its mitotic length with that of the other chromosomes. This observation may indicate that in the sperm head the heterochromatic and euchromatic regions are in the same condition (i.e. that there is no heteropycnosis at this time). If Muller is correct in his assumption that the Y really contains very few genes, then Kaufmann and Demerec's results also suggest that chromosome breakage takes place in the internodes between genes, rather than in the limits of the genes themselves. It is known that structural rearrangements of sections of chromosomes (which result from breakages followed by reunion in a new sequence) are often accompanied by apparent mutations. It now seems probable that these are true position-effects (due to the gene having an altered function in a new situation) rather than the result of 'injury' to the gene in the process of breakage. But the whole problem is still a matter for further investigation.

In a number of species, both of animals and plants, heterochromatic chromosomes may be present in some individuals but not in others. Such chromosomes are referred to as *supernumeraries*, since they are not essential for life. Thus in Maize the so-called B-chromosomes vary in number from none to about 20 without apparently affecting the plant in any way. Since the presence of an extra Y in a female *Drosophila* leads to an increase in the amount of nucleotides in the cytoplasm of its eggs (Caspersson and Schultz¹³), it seems probable that supernumerary chromosomes, in common with all heterochromatic regions, play a part in regulating the general nucleic acid metabolism of the cell and hence of the organism, although they are 'inert' in the ordinary genetic sense.

In the bed bug it has been shown that the number of heterochromatic 'X-chromosomes' varies from 3 to 15 (Slack⁴⁸; Darlington¹⁸). It is probable that only two of these really have a sex-determining function, the others being inert supernumerary chromosome-fragments which play no part in sex determination. They may, however, have some effect on the animal by making more nucleic acid than would otherwise be present.

A special type of heteropycnosis is found in the males of certain scale insects, where one entire haploid set of chromosomes is positively heteropycnotic (Schrader^{43, 44}; Hughes-Schrader²⁶). This heteropycnosis of half the chromosomes exists in all the somatic tissues as well as during spermatogenesis. It is not known whether the set which is heteropycnotic is the maternal or the paternal one, or whether it may include members of both. Most probably it represents the entire set derived from one parent. In that case it must have been 'pre-conditioned' (by passing through the egg or sperm) in such a way as to render it heteropycnotic in a male individual but not in a female. Clearly, heteropycnosis cannot be an indication of genetical inertness in these scale insects.

d. Endopolyploidy and Salivary Gland Chromosomes

It has been known for a long while that many of the somatic nuclei of insects are normally polyploid, but the extent and nature of this phenomenon has only been realized in recent years. Most of the tetraploid, octoploid, 16-ploid, &c., nuclei which occur in the hypodermis, fat body, oenocytes, &c., have been derived from diploid nuclei by a process of repeated division of the chromosomes without any true mitosis. The cells which undergo this 'endopolyploidy' seem, in fact, to have lost the power of division, but they go on increasing in size and from time to time their chromosome number is doubled. The chromosomes are usually in a loose 'flocculent' condition, so that they can only be counted with considerable difficulty. They periodically go through more or less normal prophase stages (Painter and Reindorp⁴⁰), but no spindle is formed, and the split halves of the chromosomes gradually separate from one another without the nuclear membrane breaking down. If we regard this process of 'endomitosis' as a degenerated form of mitosis, it is interesting to note that the series of changes characteristic of prophase (gradual accumulation of nucleic acid on the chromosomes) is still retained—a fact which tends to confirm the view that a certain minimum amount of nucleic acid is essential to chromosomal reproduction, and that the quantity present in the 'resting stage' is below that minimum. A good example of endopolyploid somatic nuclei is shown in Plate 1, Fig. 3.

The most thorough study of endopolyploid nuclei has been carried out by Geitler^{21, 22, 23} in the Pond-skaters of the genus *Gerris*. The diploid set of the male consists of 20 autosomes and an X which is heteropycnotic. The individual chromosomes can be counted in many of the somatic resting nuclei, and in some species the X's can be made out very clearly on account of their heteropycnosis. Geitler found that particular tissues showed characteristic degrees of polyploidy. The cells of the tracheal epithelium were diploid or tetraploid, those of the

fat body tetraploid, octoploid, and 16-ploid, those of the oenocytes up to 128-ploid, while in the salivary glands Geitler found enormous branching nuclei which he estimated from their volume to be 512-ploid, 1,024-ploid, and even 2,048-ploid. It was naturally impossible to count the chromosomes in the largest nuclei (a 2,048-ploid nucleus should have contained 22,528 chromosomes!) It seems likely that the great branching nuclei in the spinning glands of the Lepidoptera and Trichoptera (Vorhies⁵¹) are of the same general type as those of *Gerris*. As the chromosome number is repeatedly doubled, the nucleus increases in size and spreads out in a ramifying manner in the cytoplasm. Why it should do this rather than remain spherical is not clear. It has been suggested that the irregular shape of the nucleus has a functional significance, since it greatly increases the surface area of the nuclear membrane. Alternatively, it is possible that the cytoplasm (which must contain large quantities of silk) is not homogeneous, and that the nucleus extends through the more fluid regions, avoiding masses of material which are about to be secreted.

To what extent endopolyploidy occurs outside the Insecta is still doubtful. It would be of considerable interest to know if it exists in the vertebrates, but the condition of the somatic resting nuclei is not suitable for even an approximate estimate of the numbers of chromosomes they contain. But it has been shown by Jacobi²⁷ that certain mammalian tissues contain nuclei of several size-classes, which may correspond to different degrees of polyploidy.

A special kind of endopolyploidy appears to be responsible for the production of the 'salivary gland' chromosomes in the Dipterous flies. These chromosomes are entirely different in appearance from those of the salivary glands in other orders of insects. They appear to be due to repeated multiplication of the original chromosome, without any separation of the resulting threads which remain in contact with one another so that the final result is like a many-stranded rope. It is not known whether the number of successive divisions is the same from end to end of the chromosome, but in *Chironomus* at any rate (Plate I, Fig. 4) the diameter of the salivary chromosome varies from one region to another so much as to suggest that it is not. The name 'salivary gland chromosome' is really a misnomer, since chromosomes of this type are also found in many other somatic tissues in the Diptera. Thus Makino³² found that in *Drosophila virilis* the nuclei of the oesophageal epithelium, the glandular cells of the proventriculus, the cells of the intestinal epithelium, of the Malpighian tubules, and of the fat body all contained chromosomes which approximated to the 'salivary gland' type. There seems to be a complete series of gradations in these and other tissues between normal somatic chromosomes and salivary gland ones, the intermediate stages having undergone varying degrees of multiplication.

So far no chromosomes of the 'salivary gland' type have been found outside the Diptera. The reason for this is not clear, but it may be connected with the phenomenon of 'somatic pairing' which is likewise characteristic of the chromosomes in that group, and is not found elsewhere (at any rate not to the same extent). Since most of the more refined modern cytogenetic methods of analysis depend on the use of salivary gland chromosomes it will be seen that the choice of *Drosophila* as material for genetical work was a far more fortunate one than was realized before 1935 (when salivary chromosomes first came into general use in genetical laboratories).

In addition to being much thicker than ordinary mitotic chromosomes the salivaries are also much longer. In *Drosophila* they are about 100 times the length of metaphase chromosomes (Pätau⁴²; Bauer^{6,7}), while in *Sciara* they are stated by Buck¹⁰ to be about 80 times longer. Owing to their great length they can only find room, even in the very large nuclei in which they occur, by being rolled up into a fairly close tangle, which fills practically the whole of the nuclear cavity. In making preparations of these chromosomes it is usual to crush the glands in a solution of 'aceto-carmin' under a cover-glass, so that the threads are stretched and spread out flat.

Each individual chromosome in the salivary gland nuclei is closely paired with its homologous partner. This means that the threads appear to be present in the haploid number, rather than the diploid one. The pairing is probably merely another example of the phenomenon of 'somatic-pairing' which, as we have already seen, is characteristic of all Dipterous chromosomes. As a matter of fact the closeness of pairing varies somewhat throughout the Diptera; in the salivaries of some genera it is as intimate as meiotic pairing, while in others it is much looser, so that the homologous chromosomes are only in actual contact at a few points, the remaining regions being loosely approximated or wound round one another.

Each individual salivary chromosome appears under the microscope as a cross-striated structure consisting of alternate light and dark bands (or disks if we think of them as running right through the thickness of the chromosome, as they appear to do). It has been proved by staining methods, ultraviolet spectroscopy (Caspersson¹²), and micro-incineration (Barigozzi^{4,5}) that the dark bands are rich in nucleic acid, while the internodes between the bands are practically free of this substance. The individual bands vary in thickness, the broadest ones being visible in the living cell, while the finer ones can only be made out in stained preparations under the most favourable optical conditions (Bridges^{8,9}). The bands are constant in position for any one species (apart from the occasional presence of inversions or other structural rearrangements) so that it is possible to construct 'maps' of the chromosomes in which

each band is referred to by number. These maps constitute the basis of most modern cytological work on *Drosophila*.

The total number of bands cannot be stated with certainty, since many of them are so fine as to be almost beyond the power of resolution of the microscope, while some of the thicker ones may actually consist of several bands situated so close together (i.e. with such short internodes between them) that they appear as a single one. In Bridges's last⁹ map of the X-chromosome in *D. melanogaster* over 1,000 bands were detected. If the 'IInd' and 'IIIrd' chromosomes each contain about 2,000 bands, and the tiny 'IVth' about 50, the total number must be somewhat over 5,000.

In *Drosophila* the heterochromatic regions round the centromeres are all fused together in the salivary nuclei so as to form a body known as the chromocentre. The Y-chromosome is also associated with this body and the nucleolus is attached to it. All the heterochromatic regions are relatively much shorter in the salivary chromosomes than in ordinary mitotic ones: they contain a few bands which only stain rather faintly. In many species of Diptera there is no chromocentre, the salivary chromosomes being free. The presence or absence of a chromocentre seems to depend more on the closeness of somatic pairing in the species in question rather than on the presence or absence of heterochromatic regions, since many of the species without a chromocentre have extensive heterochromatic regions, either intercalary or terminal.

e. Spiral Structure

It is now generally agreed that the cylindrical appearance of chromosomes (or chromatids) at metaphase is a deceptive one, each apparently solid cylinder being really a tightly coiled spiral. Thus the thread along which the genes are spaced is in reality a good deal longer than the metaphase chromosome. The spiral is a 'compact' one, in which there is virtually no space left between the gyres or in the centre of the coil, so that the true structure can only be made out by utilizing special techniques which artificially separate the gyres.

No spiral structure can be observed in salivary gland chromosomes, except that frequently the homologues are loosely wound round one another. It would seem that the absence of spiralization is one of the reasons for the great length of the salivaries, as compared with ordinary mitotic chromosomes. It can hardly be the only reason, however; the disparity in length is too great.

Spiral structure was first observed by Baranetsky² in 1880 in the chromosomes of the plant *Tradescantia*, but it was not until recently that it was realized that this is the normal structure of all late prophase and metaphase chromosomes. The usual methods of fixation, sectioning, and staining are in fact unsuitable for revealing the spiral structure

of chromosomes, except in particularly favourable cases; in plant material it is now usual to employ for this purpose some smearing technique or special chemical treatment prior to fixation. These methods either stretch the chromosomes somewhat roughly or in some other way separate the gyres of the spiral. While the usual techniques undoubtedly give one a very accurate impression of the chromosomes as they appear in the living cell, the special techniques used for demonstrating spiral structure give rise to an artificial picture which may be described as a *dissection* of a chromosome, since it reveals the internal structure.

The whole cycle of mitosis can be regarded as a cycle of spiralization and un-spiralization, a new coiling system being formed in the protein framework at each division. Usually the coils of one mitosis persist to some extent through the resting stage, so that they can still be recognized as loose 'relic spirals' in the prophase of the next division. These 'relic spirals' can be seen very clearly in the spermatogonia of some of the long-horned grasshoppers (Plate 2, Figs. 1 and 2). They disappear before metaphase, the new coiling system (which is invisible in ordinary preparations) having by that time fully developed within the chromosome. The number of coils per unit length naturally depends on the diameter of the chromosome, to which it is inversely proportional (White⁵⁴). Thus the X-chromosomes in grasshoppers have far fewer coils when positively heteropycnotic than when negatively heteropycnotic. During prophase the number of coils must decrease as their amplitude increases until at metaphase, when chromosomes are compact and have a smooth, even outline, the number of coils in each chromosome is $2l/d$, where l is the length and d the diameter.

It was for some years a matter of controversy whether the direction of coiling (right- or left-handed) was constant or at random for any particular chromosome or chromosomal region. There is now overwhelming evidence (Matsuura^{33,34}; Nebel³⁹; White⁵⁴) that it is at random. Frequently the direction of coiling may be reversed at the centromere, and reversals have also been observed by some workers at other points along the length of the chromosome. The fact that the direction of coiling in a particular chromosome is at random is, incidentally, evidence that a new set of spirals is formed at each mitosis. If the same spiral persisted from division to division we should expect that all the descendants of a single chromosome should be coiled in the same direction. It has been shown, however (White⁵⁴), that a cyst of 32 or 64 grasshopper spermatogonia, which are all derived from a single ancestral cell, may contain an approximately equal number of left-handed and right-handed X-chromosomes.

Theoretically, two kinds of spiral are possible; these have been called the *orthospiral* and *anorthospiral* types by Kuwada³¹. To produce an

anorthospiral it is merely necessary to take a thread or piece of wire, fix both ends firmly in a pair of clamps and twist the middle part around a cylinder or rod of some kind. It will be found that in addition to the obvious spiral produced in this way the substance of the thread is itself twisted in a 'compensating' spiral of the opposite sign (i.e. right-handed if the main spiral is left-handed). In an *orthospiral* there is no compensating twisting of the substance of the thread, and such a spiral can only be formed in a thread with at least one end free to rotate. Darlington^{16,17} has assumed that chromosomes have an *anorthospiral* structure, but there is no direct evidence for this view.

Kuwada and his co-workers are of the opinion that in the meiotic chromosomes of certain plants the thread which forms the spiral is itself spirally coiled. There is thus a 'major' and a 'minor' spiral, as in the 'coiled coil' filaments of certain electric lamps. It would seem that the minor coil is an anticipation of the spiral structure of the second meiotic division, and that a double spiral is probably present in most chromosomes which undergo two divisions in rapid succession. Naturally, the 'minor' spiral is much more difficult to demonstrate than the 'major' one, but in a few instances convincing photographs of it have been published.

iii. PARTITION OF CHROMOSOMES

a. Mitotic Splitting

A controversy has existed for several years as to the exact stage in the nuclear cycle at which chromosomes first split longitudinally. It is agreed by all workers in this field that at prophase the chromosomes are longitudinally divided in preparation for the separation of the daughter chromosomes at anaphase. Thus a prophase chromosome consists of two spiral threads or *chromatids*, which lie roughly parallel to one another and are joined together at the centromere. These spiral chromatids may be pressed into one another so that no space can be seen between them or they may be separated so that there is a visible split down the length of the chromosome. The controversy to which we have referred related to the exact stage in the nuclear cycle at which this split occurs. Darlington and his fellow-workers believe that it takes place during the resting stage, when the chromosomes are not in a suitable condition for accurate observation, being more or less 'unfixable'. Thus they believe the telophase chromosome thread to be always single, the prophase one always double, whereas other authorities maintain that telophase chromosomes are double, prophase ones quadruple, the split having taken place at some time in the previous division. One worker (Nebel³⁹) actually maintains that telophase chromosomes are quadruple, the split corresponding to each mitosis having taken place two mitoses before. The protagonists on both sides of this polemic

seem to have tacitly assumed that whichever opinion was true must also be true in all types of cells. This seems unlikely—at any rate we have no right to suppose that the split always takes place at the same stage of the nuclear cycle, when it is known that in some cells the resting stage is very prolonged while in others there is a direct transition from the telophase of one mitosis to the prophase of the next. It is probable that the importance of this disagreement has been magnified, since it is generally admitted that a split may in fact exist long before it is visible under the microscope.

That chromosomes may be caused by experimental agencies such as X-radiation and abnormal temperatures to undergo an extra split, so that they are quadruple at stages of mitosis where they would normally be double, has been shown by various workers (White⁵³; Barber³). It is possible that this condition usually results from the suppression of a mitosis which would normally have occurred, the split which would have corresponded to that division not being inhibited. Since the chromatids in such a nucleus fall apart eventually the condition is akin to endopolyploidy and may be regarded as an experimentally produced endomitosis.

In certain animals some of the chromosomes are eliminated during the cleavage divisions. This is notably the case in flies of the genus *Sciara*, where certain large chromosomes (known as the 'limited' chromosomes) are eliminated from all those nuclei which are destined to give rise to the somatic tissues (du Bois²⁰; Metz³⁶). The centromeres of these chromosomes seem to function normally up to and during the metaphases of these divisions, but it appears that the chromosomes have not split, so that when the spindle elongates at anaphase the limited chromosomes cannot pass to either pole, since they are undivided. They are thus trapped in the middle part of the spindle, and do not become included in either of the daughter nuclei. It is, of course, possible that a split does form in these 'limited' chromosomes, but that the chromosomes are in some unknown way prevented from separating from one another. A similar elimination of some of the chromosomes takes place in some of the Cecidomyid flies (Kraczkiewicz^{29,30}).^{*} A failure of the chromosome thread to divide has been induced in certain plant chromosomes by the application of unusually low temperatures (Darlington and La Cour¹⁹). In this instance the failure to divide occurs only in certain chromosomal regions which are deficient in nucleic acid in the chilled material. This observation suggests that the normal process of 'splitting' depends on the presence of a certain minimum quantity of nucleic acid, and that when a sub-minimal amount is present the

* The elimination of the ends of the 'germ-line' chromosomes of *Ascaris* is probably due to an entirely different cause, namely, to the fact that when the chromosomes undergo fragmentation these ends are left without centromeres.

split will not occur (it will be remembered that the evidence from endopolyploid nuclei also points to this conclusion).

b. Structural Rearrangement

We have already referred to the fact that chromosomes may be broken across transversely by means of X-rays. This breakage is usually followed by a spontaneous reunion of the freshly broken ends. If more than one breakage is present at the same time in a single nucleus then the reunion may take place in a different way from the original one, thus leading to a new sequence of the genes. Muller believes that freshly broken surfaces are incapable (in *Drosophila* at any rate) of uniting with natural ends (i.e. telomeres).^{*} On this the new freshly broken ends possess a kind of 'stickiness' or bipolarity which the telomeres lack. It is fairly certain that any freshly broken end can unite with any other, so that all types of structural rearrangements can occur, although only chromosomes containing one centromere and two telomeres are viable. (There is one important exception to this rule, namely, that ring-shaped chromosomes—in which both telomeres have been lost and the two ends have fused—are viable in *Drosophila* and in some other organisms, although they suffer from various mechanical disabilities at mitosis.)

It is easy to understand why portions of chromosomes lacking centromeres are inviable. Not being attached to the spindle at mitosis they simply float about in the cytoplasm and usually fail to become included in either of the daughter nuclei at telophase. A chromosome which remains for any length of time in the cytoplasm always seems to die eventually, being probably dissolved by enzymatic action. Why telomeres are necessary to the life of the chromosome is not so clear. Strictly speaking, they are not necessary, since ring-shaped X-chromosomes are viable in *Drosophila*, as we have already seen. But there is no undoubted record of a chromosome with a freshly broken end surviving for more than a few cell generations in *Drosophila*. Thus it would appear that the telomeres should be regarded as 'protecting sheaths' over the end of the chromosome, which in some way insulate it from the surrounding medium and are not necessary for the life of an endless ring-chromosome. It should be emphasized that the existence of telomeres is inferred from the results of experimental breakage of chromosomes—they have not been actually seen as the centromeres have. Nor is it by any means certain that the telomere concept is valid in all organisms. Thus in *Ascaris megalocephala*, where the germ-line chromo-

^{*} Some other workers claim that terminal deficiencies, inversions, and translocations all occur and may in special cases be viable. The point at issue is whether the rearrangements studied by these authors were strictly terminal and not subterminal.

somes spontaneously break up into a large number of smaller bodies in the somatic cells, it is fairly clear that the concept does not apply (unless we suppose that potential telomeres already exist at intervals along the length of the germ-line chromosomes before fragmentation). In maize, too, it appears that terminal deficiencies and inversions occur quite frequently in the progeny of irradiated material (McClintock³⁵).

Although 'structural rearrangements' can be induced by irradiation with X-rays, it is known that they also occur spontaneously from time to time. In natural populations of *Drosophila* species, several different types of gene sequence frequently exist side by side; such a condition can only be due to structural rearrangements having taken place in the course of evolution. Moreover, the visible differences which exist between species in regard to chromosome shape and size must be the result of structural rearrangements which have taken place in phylogeny. Just as in the case of the 'natural' mutations, it is not known what causes 'spontaneous' chromosome breakage. It has been shown that the natural mutation rate is far greater than could be accounted for if it were entirely due to cosmic radiation or to traces of radioactive substances in the earth's crust. As far as the spontaneous breaks are concerned, such a calculation has not been made, since their rate of occurrence has never been measured accurately.

It will be apparent that there is an obvious similarity between structural rearrangements and the phenomenon of *crossing over* which normally takes place at meiosis. In both instances breakage of chromosome threads is followed by reunion in a new way. The breakages are believed to occur in the internodes between the genes, and when the process of joining has been completed the chromosome is 'as good as new'—i.e. there is no visible indication of the point of union. Whether there is only one point between two successive genes where breakage can take place, or whether there may be several, is unknown.

It has been known for some years that the number of mutations produced in *Drosophila* sperm by a given X-ray dosage is directly proportional to the number of ionizations produced, and is independent of the wave-length. Moreover, the irradiation may be crowded into a few seconds or spread over a period of days: so long as the total dosage is the same the number of mutations will be identical. Thus the relation between mutation and dosage obeys the Bunsen-Roscoe law and is a linear one. This information strongly suggests that mutations are produced by single ionizations.

When we come to consider the gross structural rearrangements (translocations, inversions, &c.) we find that the number of rearrangements produced is proportional to the $\frac{3}{2}$ power of the dosage. As in the case of mutations, the wave-length and time of exposure are again immaterial to the final result, provided that the dosage is kept the same.

The interpretation of this exponent of 1.5 has given rise to a certain amount of confusion. If structural rearrangements are due to single rare accidents then we should expect a linear relationship between dosage and number of rearrangements. On the other hand, if breakage and reunion are to be regarded as independent accidents one would expect to find that the number of rearrangements would be proportional to the square of the dosage. It is probable, however (Muller³⁷), that the failure to obtain an exponent of 2.0 is due to the following factors: (1) the so-called 'saturation effect' (a certain number of multiple rearrangements are mistakenly counted as simpler rearrangements), and (2) the fact that there is an increased chance for these multiple rearrangements to give rise to inviable chromosomes (lacking centromeres, or possessing two centromeres, &c.). Thus the exponential relationship found is probably not irreconcilable with the view that chromosome breakages in the sperm are produced by single ionizations and that these breakages accumulate until fertilization, at which time the broken ends have a chance to rejoin.

It has been known for some time, however, that in irradiated material 'minute' rearrangements (inversions, deletions, &c., of very short segments comprising at most three or four bands in the salivaries) are disproportionately common. That is to say, if there are two breaks in a chromosome the second is more likely to be very close to the first than can be explained by chance. Moreover, it has been found that these 'minute rearrangements' do not obey the '1.5 rule', since their number is directly proportional to the dosage employed in the experiment. There are thus good grounds for regarding the 'minute' rearrangements as somewhat different in their nature from the 'gross' ones. It has been suggested that one ionization may, in fact, produce two breaks in the same neighbourhood. The idea that 'minute' rearrangements are multiple effects of single ionizations explains their two anomalous characteristics (abnormally high frequency and linear relationship to dosage). It will be obvious, however, that if rearrangements are on a sufficiently small scale to be invisible in the salivary chromosomes it must be exceedingly difficult to distinguish them from gene mutations. In fact, the view has been put forward by some cytogeneticists that there is no real difference between minute rearrangements and gene mutations, the latter being simply inversions, translocations, &c., on a sub-microscopic scale. This view was, of course, strengthened by the discovery that both types of effect were directly proportional to the dosage used in the experiment. Moreover, it is clear from recent salivary gland work (e.g. that of Slizynski⁴⁹) that many of the so-called 'lethal' mutations are in fact minute structural changes (usually deletions of a few bands). But it now seems likely that at any rate the majority of the visible (i.e. non-lethal) mutations are really different in kind from the minute

rearrangements. The most decisive evidence on this point is derived from recent work on the production of mutations by the use of ultraviolet radiation (Muller and Mackenzie³⁸; Muller³⁷) and a comparison of these data with those obtained in the X-ray experiments. It was found in the course of this work that the ultraviolet radiation gave rise to a large number of 'visible' gene mutations but not to any 'gross' rearrangements. An X-ray dose sufficient to have caused the same number of mutations would have caused about seventy-five 'large-scale' translocations. These results are interpreted by Muller as evidence for a real difference between gene mutation and chromosome breakage. Similar work by Stadler and his co-workers on maize seems to point in the same direction.

c. Genes and Crossing Over

We have dealt with these questions of mutation and chromosome breakage at some length because of their bearing on the more general problem of the molecular structure of chromosomes and the nature of the gene. The whole subject is complex, but has been fully discussed in an admirable review (Muller³⁷) in which references to the original papers may be found.

Up to now we have spoken of chromosomes as if they were made up of alternate 'genetic' and 'non-genetic' segments (genes and internodes). This is the classical view, due to Morgan, Bridges, Sturtevant, and Muller. It has recently been severely criticized by Goldschmidt^{24,25}. Goldschmidt goes so far as to deny altogether the validity of the gene concept. He does not believe in the existence of internodes, and regards the genetic properties of the chromosomes as the result of a continuous 'pattern' in their molecular structure. It is obvious that he has been led to this view mainly as a result of consideration of the 'position-effect' (see p. 146). The majority of modern cytogeneticists are not in agreement with Goldschmidt's theory of chromosome structure which is closely bound up with his views on evolution and with the idea that all mutations are minute structural changes. Nevertheless, it must be admitted that the discovery of many 'position-effects' suggests that genes are not quite such discrete entities as was formerly supposed, but enter into various kinds of interactions with neighbouring regions of the chromosome. One fact which seems to be evidence against Goldschmidt's view is the characteristic banding of the salivary chromosomes (which, as we have already seen, is paralleled by the 'chromomere' structure seen at the prophase of meiosis). If there is no 'functional segmentation' in the chromosome then we are left without any obvious explanation for the visible alternation of segments rich in nucleic acid with ones lacking it (or at least relatively deficient in it). It would seem therefore that it is best to retain the

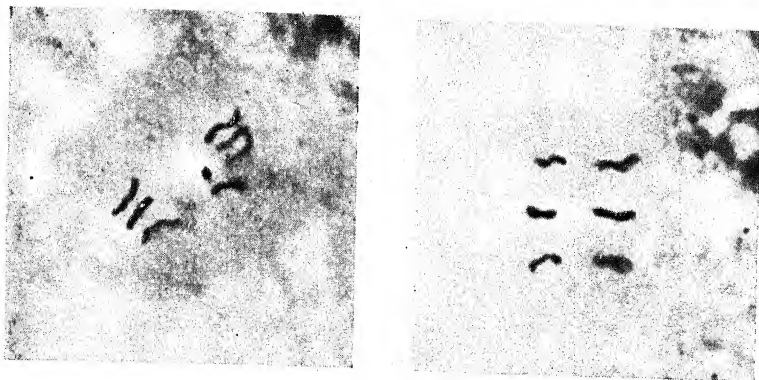
classical viewpoint for the present, perhaps in a somewhat modified form, rather than to reject it entirely as Goldschmidt now does.

If genes do really exist it is desirable that we should know something about their dimensions and should be able to estimate their total number in the chromosome set of an organism such as *Drosophila* or maize. It must be admitted that most of the earlier attempts to solve these two interrelated problems were based on what now appear to be false premisses (Muller³⁷). But such data as do exist are not incompatible with the view that the total number of bands in the salivary chromosomes is equal to the number of genes.

No analysis of the molecular structure of chromosomes can be regarded as finally satisfactory unless it provides a physical explanation of the phenomenon of chromosome pairing. None of the recent advances in cytogenetics have thrown any new light on this aspect of nuclear physiology, and the problem is as mysterious as it was when its existence was first realized. In fact many biologists seem to have become so resigned to ignorance in this respect that they hardly realize what a serious gap in our knowledge it is. Most of them do not, in fact, appreciate the physical difficulties involved in postulating the existence of specific attractions which operate at a relatively considerable distance.*

Another property of the chromosomes which is still somewhat of a mystery is the phenomenon of crossing over. It is probable that, as in the case of structural rearrangements, we are here dealing with two events, followed by reunion. Now reunion seems to be a characteristic of all broken ends of chromosomes, provided that they are not too far apart in the nucleus. But why should chromosomes (or, rather, chromatids) break at pachytene, the stage at which crossing over is known to occur? And, more especially, why should two chromatids, one derived from each parent chromosome, break at exactly the same level? It is believed by Darlington and many other workers that in meiosis the split which divides each chromosome into two chromatids takes place at pachytene, and that its origin is in some way associated with the occurrence of crossing over. Other authorities, however, maintain that a split can be detected long before pachytene. Darlington¹⁷ has put forward a theory according to which crossing over depends on a previous coiling of the homologous chromosomes round one another. This he supposes leads to a state of tension. When the chromosome splits the chromatids are unable to withstand the strain and accordingly break, allowing a relaxation of the torsional stress to take place. Several objections to this ingenious theory may be very briefly mentioned here: (1) in many species of animals crossing over normally takes place in very small chromosomes where the homologues are far too short at

* I am indebted to Dr. A. C. Fabergé for pointing out some of these difficulties to me.



FIGS. 1 and 2. Fifth cleavage division in the mite *Pediculopsis graminum* showing the elimination-bodies between the chromosomes. Fig. 1 is an early stage of anaphase, Fig. 2 a slightly later one. Preparation by Dr. K. W. Cooper. Fixation: Navashin's fluid. Staining: Heidenhain's haematoxylin.

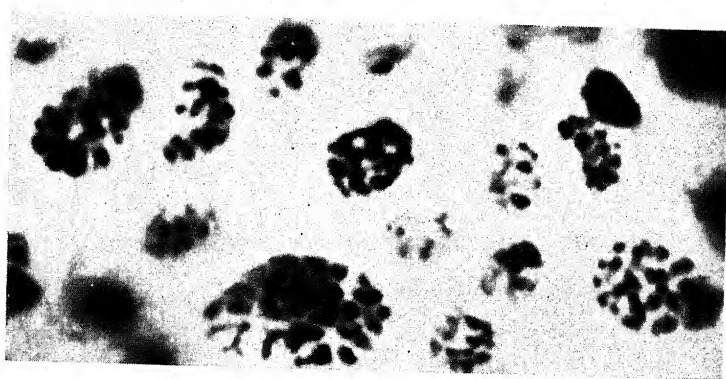


FIG. 3. Diploid, tetraploid, octoploid and (?) 16-ploid somatic nuclei in the wall of the testis of the locust *Schistocerca gregaria*. Fixation: Flemming's fluid. Staining: Gentian violet by Newton's method.

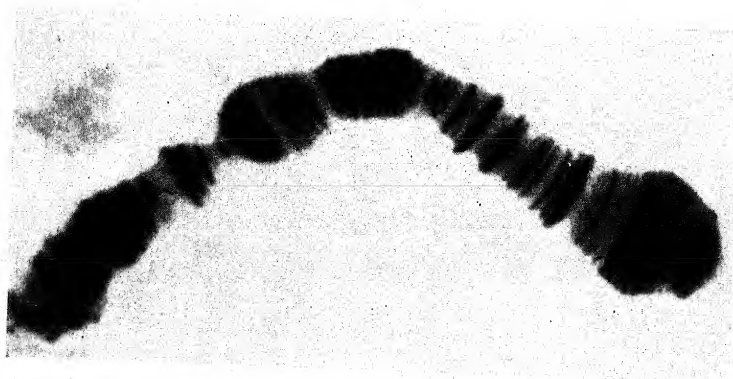


FIG. 4. Part of a salivary gland chromosome in *Chironomus* sp. Fixation and staining: 1 per cent. orcein in 45 per cent. acetic acid.



FIG. 1. X-chromosome, showing heteropycnosis and spiral structure in a spermatogonial resting nucleus of the Long-horned Grasshopper *Platycleis grisea*. Fixation: Strong Flemming's fluid. Staining: Gentian violet by Newton's method.

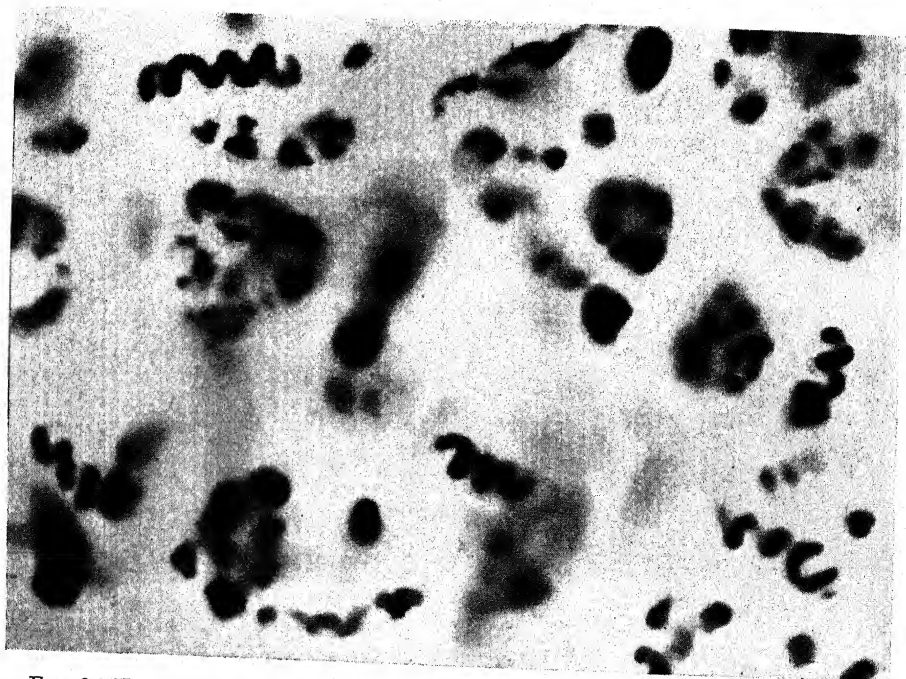


FIG. 2. X-chromosomes showing spiral structure in spermatogonia of the Long-horned Grasshopper *Insara gracillima*. Fixation: San Felice's fluid. Staining: Gentian violet by Newton's method.

pachytene (relative to their diameter) to coil round one another. Moreover, in many of these short chromosomes it can be seen that the homologues lie parallel, and not twisted together, at this stage. (2) Crossing over is almost normal in ring-shaped X-chromosomes of *Drosophila melanogaster*, in which it is very difficult to imagine how a relational coil could develop between two rings in the same manner as between two threads. It seems to the writer far more probable that crossing over is due to the alternation of heterochromatic and euchromatic segments in which the reproduction and splitting of the protein framework is not quite synchronous. If the two homologues are held together by a pairing-attraction so long as they are unsplit and repelled as soon as they are split, then a state of tension is bound to exist where a split segment meets an unsplit one. The theory receives some confirmation from the fact that in animals (such as many species of grasshoppers) in which crossing over is almost entirely restricted to certain regions of the chromosomes, those regions are usually, and perhaps always, near the 'frontiers' between euchromatic and heterochromatic segments. The idea that chromosome breakage may result from failure of the protein framework to reproduce itself at the right time, and that this failure may be due to an unusually large or small amount of nucleic acid in a particular region, is not new (see Caspersson and Schultz¹³; Schultz^{45a}; Darlington and La Cour¹⁹). But the possibility that crossing over may be due to 'errors' of this type (i.e. to slight inequalities in the rate of splitting of the chromosome at different points along its length) is one that has not received consideration up till now.

To sum up: the information which we possess about the nucleus is almost entirely confined to the chromosomes, since we know next to nothing about the nuclear sap. Our knowledge of chromosome structure is now extensive and many-sided, but it is still inadequate for us to be able to form a complete molecular diagram of their chemical structure. Nevertheless, it is clear that the original picture of a chromosome as a linear sequence of genes was merely a first approximation to the truth. They are, in fact, quite complicated organelles, whose parts (centromeres, telomeres, euchromatic and heterochromatic regions) have specific mechanical, physiological, and genetical properties. An understanding of these is fundamental to both physiological and evolutionary biology.

CHAPTER VI

MICRO-INCINERATION AND THE INORGANIC CONSTITUENTS OF CELLS

By E. S. HORNING

i. INTRODUCTION

IN 1833 the French botanist François-Vincent Raspail⁷¹ demonstrated the mineral structure of plant cells by subjecting sections of plant epidermis to a process of incineration in order to eliminate the organic matter. Since then many spasmodic attempts have been made by subsequent investigators to employ this method as a cytological procedure for determining the inorganic constituents of cells.

No significant advances were made, however, until Liesegang⁴⁴ in 1910, by selecting less primitive apparatus than previous workers, described in incinerated microscopical sections the topographical distribution of heat-resistant mineral ash of blood corpuscles in the capillaries of embryonic fish brains. He likewise described the ashed remains of such delicate structures as the retina in the developing eye, optic nerves, and individual neurones. Then followed the work of Herrera²⁷ in 1913, who applied micro-incineration to a study of various zoological and botanical problems. A few years later studies with this technique were extended by Naumann⁵², Prenant⁷⁰, and Molisch⁵⁰. Although these workers stressed the possibilities of incineration as a microscopical method, their investigations met with only moderate success until another Frenchman, A. Policard⁵⁷ in 1923 made considerable improvements in the technique. Policard introduced a suitable fixing solution and an electric quartz furnace by which the incineration process could be more adequately controlled. Scott⁸⁴ subsequently added several modifications to Policard's technique, amongst which was the examination of incinerated preparations by dark field instead of oblique illumination. These modifications established a technique by which the inorganic residues of microscopical sections could be compared with stained control paraffin sections to a remarkable degree of accuracy. The failure of previous workers had been due to their inability to find a suitable fixing fluid and devise a controlled method of incineration.

When these preliminary technical difficulties had been overcome, many workers were at once attracted by this novel microscopical technique and it was enthusiastically applied to an examination of material covering a wide biological field. Formerly the only available

method of determining the inorganic contents of cells was by a chemical analysis of tissues in bulk: a technique hardly adaptable for bacteria, inclusion bodies, protozoa, and other individual cell types. Papers describing the mineral components of inclusion bodies associated with certain virus diseases, bacteria, and protozoa soon appeared, while pathologists likewise applied micro-incineration to an extensive study of normal and diseased tissues.

One advantage of incineration is that it permits a microscopic examination of the relative topographical distribution of mineral ash within individual cells, giving an indication of its probable distribution in the living tissues and organs of the body⁸⁸. Certain technical difficulties occur, however, owing to the exceedingly small concentrations of the inorganic residues involved in thin sections suitable for microscopical examination. The main problem confronting investigators has been to discover analytical means by which quantitative and qualitative estimations of the inorganic constituents of incinerated sections could be determined.

From 1928 onwards the optical appearance of certain heat-resistant minerals in incinerated preparations examined in dark field, polarized, and ultraviolet light, was recorded. This method, frequently used in conjunction with microchemical tests, was in some respects unsatisfactory and uncritical. It became obvious from the numerous papers published at that time that the interpretation of the visual appearances of the minerals forming the ashed residues was hardly sufficient to establish their chemical identities. The possibility of complex combination of the inorganic salts occurring during the incineration of the tissues was apparently not fully appreciated. Such limitations of micro-incineration as a histochemical technique were stressed by Scott⁸⁹ in a critical review published in 1933. Nevertheless, even without accurate chemical identification of ash residues, many interesting observations resulting from the use of micro-incineration have been recorded, which will form the subject of a later discussion.

It was realized that if quantitative and qualitative means of identifying the inorganic constituents of cells and tissues could be discovered, an entirely new approach to cell physiology could be made. Researches in this direction were undertaken with the aid of the magnetic electron microscope, spectrographic analysis, and a photo-electric process. So far the most promising results were obtained in 1939 by the successful and ingenious application of a modification of the electron microscope, by which Scott and Packer⁹⁵ have succeeded in localizing mineral elements *in situ* in ashed tissue sections.

A short account of the technique of micro-incineration, its application to biological and pathological problems, and a survey of the results obtained will now be undertaken.

ii. TECHNIQUE OF MICRO-INCINERATION

a. *Methods of Fixation.*

The most universal and convenient method of fixing fresh tissues for micro-incineration is that originally described by Scott⁸⁵, in which small pieces of tissues are fixed for 24 hours in a solution containing 9 volumes of absolute alcohol to 1 volume of neutral commercial formalin. In order to ensure complete dehydration and to remove the formalin, all material is then passed through several changes of absolute alcohol, prior to clearing in xylol and embedding in paraffin. Absolute alcohol was selected as a fixative, since very few of the inorganic constituents, common to protoplasm, are soluble to any great extent in this fluid. Owing to the fact that absolute alcohol is such a poor histological fixative and in order to minimize cell distortion, formalin was added to the alcohol. Scott⁸⁵ found that analysis of the formol-alcohol solution, subsequent to its use for the routine fixation of tissues, failed to give any positive reactions for K, Na, Mg, or Ca. It may be assumed, therefore, that the fixation process does not extract appreciable amounts of inorganic substances before incineration, although it is possible that, during the penetration of the fixative, some shift in the localization of individual inorganic substances may occur in the cells and tissues.

According to Alexander and Myerson² tissues that have been preserved for several weeks, either in 10 per cent. formalin alone or in 95 per cent. alcohol, can be used for incineration, but they report that less brilliant ash preparations were obtained. Distortion owing to tissue shrinkage was commonly found. Gage²¹ advised fixation in a 3 per cent. aqueous solution of potassium bichromate with the addition of 10 per cent. neutral formalin, and suggests even the use of Helly's fluid. Washing the material in running tap-water and repeated immersion in 70 per cent. alcohol is required to remove the chromium. Experience has shown that fixation in either of the above solutions fails to give results as good as those obtained by the use of alcohol-formalin. For obvious reasons it is advantageous to avoid aqueous fixing fluids containing either chromium or mercury when preparing tissues for micro-incineration.

Alexander and Myerson² recommend the use of frozen sections of fresh material no thicker than 10 μ , providing the sections are passed through graded alcohols and ether prior to incineration. As is well known, it is difficult to control the thickness of frozen sections, and the subsequent loss or distortion of the soluble inorganic salts, by treatment with low-grade alcohols, must result in loss of detail after ashing. This method, however, is convenient for a rough quantitative estimation of ash distribution in pathological preparations.

The Altmann-Gersh freezing-drying technique undoubtedly has

proved the most successful method for the preparation of thin sections of animal tissues suitable for micro-incineration. In fact by this process, which has been modified by Gersh²³, Bensley and Gersh⁷, and others, it has been possible to make most important contributions to the study of cytology as a whole. Among its many advantages are the instantaneous method of fixation and the sudden cessation of all metabolic activities and shifting of substances within the cells and tissues immediately before the fixation process is carried out²⁹. The technique permits, moreover, the preservation of mitochondria and other cytoplasmic inclusions and also of vitally stained preparations which are so often difficult to preserve in routine fixing solutions. The absence of cell shrinkage and no appreciable diffusion or displacement of the cell constituents make the technique very desirable for combination with micro-incineration.

In 1932 Gersh²³ developed the Altmann method of freezing animal tissues with liquid air and of subsequently drying them *in vacuo* at low temperatures. In the dried state such material is suitable for cutting hand sections or for direct infiltration with paraffin so that the sections are prepared from material which has not come into contact with any of the reagents and solvents commonly used in microscopical techniques. Scott²⁹ was one of the first to apply this method in conjunction with micro-incineration. He found, however, that while tissues treated by the Altmann-Gersh technique produced an ash residue showing cytological structure, the results were not comparable with those obtained by the routine alcohol-formalin fixation. While at the University of Chicago the author had the opportunity of making a similar comparison, and confirmed Scott's statement. Scott²⁹ also found that the distribution of inorganic salts, as seen in incinerated tissue sections, varied according to the temperature of dehydration. At that time Gersh²³ recommended that for histological purposes tissues should be dehydrated at -20°C . Scott pointed out that the temperature selected by Gersh as suitable for carrying out dehydration of the frozen tissues was above the eutectic point (-21°C .) of such universally distributed salts as sodium chloride. It was also found that freezing by liquid air produces in tissue cells a solid mass of ice crystals in equilibrium. Disturbance of this equilibrium by warming the frozen tissues above eutectic point would lead to diffusion and shifting of ions during the period of dehydration. Scott²² then showed that this objection might be overcome by dehydrating tissues at temperatures ranging from -38°C . to -78°C . The incineration of sections from this material yielded results closely comparable with those obtained by alcohol-formalin fixation. Further experiments led Scott²⁹ to recommend the use of alcohol chilled to -177°C . instead of liquid air. More recently Hoerr²⁹ has confirmed the fact that the rapidity with which tissues are frozen determines the

quality of the cytological fixation. He has stated that 'if the tissue is not frozen at the optimum rate, so much of the free water in the tissue may freeze out that the tissue is blown up by large ice crystals and after drying presents an extremely vacuolated appearance'. Hoerr has recommended more rapid freezing of tissues by means of pentane chilled to -131°C . or iso-pentane chilled to -195°C ., followed by dehydration at a temperature of -30°C . He has criticized Scott's use of chilled alcohol on the ground that alcohol at -177°C . is so viscous that the freezing is not as rapid as that obtained by the use of pentane. In addition it was shown to be advantageous to allow tissues to warm up very slowly after the initial freezing to the temperature of dehydration.

Scott and Packer⁹⁵ expressed the opinion that provided material is previously prepared by the pentane modification of the freezing-dehydration method, there exists little chance of 'any perceptible shift in the cellular location of the inorganic constituents'. Their opinion was based upon tissues examined by the electron microscope after incineration. More recently Scott⁹² has returned to the use of liquid air and provided the material is dehydrated *in vacuo* at -63°C . and subsequently infiltrated with water-free paraffin, it was concluded that no appreciable shift occurred in the inorganic salts, the addition of water from external sources having been eliminated.

Attempts to apply the freezing-drying technique to plant tissues did not give satisfactory cytological results at first. The majority of plant cells possess higher mineral salt concentrations and their water content is higher than in animal cells. Goodspeed and Uber²⁴ have introduced new modifications of the technique to overcome these difficulties, and they have recommended particularly a slower rate of dehydration for plant tissues.

b. *Micro-incineration.*

In order to obtain the most satisfactory cellular details in ash residues, sections should be no thicker than 5μ . Series of sections should be cut in which alternate ones are kept for incineration, the remainder being mounted and stained in the routine manner for controls. Only the best quality glass slides are suitable for mounting sections for incineration. The sections should be smoothed out by using either absolute alcohol or liquid petrolatum. Care must be taken to avoid contact with water which might involve a loss or disarrangement of the soluble inorganic salts. The contamination of sections by dust must be avoided.

The glass slides, on which the sections are mounted, are placed on small quartz plates (see Pl. 1, Fig. 1) which lie horizontally in the furnace. The type of electric quartz furnace recommended is illustrated on Plate 1. A series of calibrations on the rheostat are used to mark

a range of temperatures suitable for different types of material. Although Tschopp¹⁰² advises passing oxygen through the furnace during the incineration, and Schultz-Brauns⁶¹ employs nitrogen, the air currents set up by the heating of the quartz tube appear to be sufficient to provide ample oxygen for incineration. The use of gases enables incineration to take place at lower temperatures whereby the greater proportion of chlorides in the ash may be retained. Policard⁶¹ for this reason has used a mixture of nitrogen together with a small concentration of oxygen in order to bring about a more rapid oxidation. Provided the preparations are not incinerated too rapidly, satisfactory results are nearly always obtained by burning in air. The temperatures required to burn off organic matter vary with different tissues. Thus certain types of neoplastic growths, tar tumours in particular, and mammary glands fixed during functional activity, remain carbonized longer than the majority of normal tissues. Protozoa and tissue cultures incinerate very rapidly. Generally speaking, the incineration of normal tissues should extend over periods ranging from 45 to 55 minutes, during which the temperature should be increased by approximately 70° C. every 5 minutes to reach a maximum temperature of 650° C. Tissues containing high concentration of collagen or elastic fibres tend to shrink when the temperature is first raised to 70° C., but this can be avoided if the rise in temperature is spread over a longer interval. Similarly, thin sections of brain require a more gradual rise in temperature during incineration to avoid distortion. Scheid⁷⁹ has found that thick brain sections incinerate more successfully if the phospholipids are first extracted with suitable solvents.

When the incineration is completed, the slides are allowed to cool slowly before removal from the furnace. A cover-slip is then placed over the ashed preparations, the edges of which are sealed with warm paraffin, thus permitting the use of an oil immersion objective if required. The sealing also prevents the absorption of moisture and efflorescence of the ash. The incinerated sections should be examined at once by direct illumination to ensure that complete incineration has been achieved. Carbon appears black or brownish by transmitted light and consequently areas in a section which have not been completely incinerated can be easily detected. The ash distribution of the incinerated section is then viewed by dark field illumination. With this method the contours and relative densities of the ash are revealed with remarkable brilliance. A dark field condenser allowing a rapid change from dark field to direct illumination will be found useful for the examination of the incinerated sections and the stained control preparations. High-power investigation of ashed sections can be undertaken with an apochromatic homogeneous oil-immersion objective provided an iris diaphragm is fitted within it.

iii. THE IDENTIFICATION OF HEAT-RESISTANT MINERALS IN MICRO-INCINERATED SECTIONS

The precise chemical identification of inorganic substances *in situ*, within individual incinerated cells, has proved the most difficult aspect of the technique. Unfortunately, the inorganic ions in cells often form complex combinations with organic substances and doubtless certain complex chemical reorientations must occur during the process of incineration. Moreover, it has been calculated that calcium, for instance, is present in the order of 1×10^{-13} grammes per cell of 1,000 cubic μ ⁸⁹. Such a minute concentration falls outside the limits of many histo-chemical tests. Recent analytical methods which are still in an experimental phase, however, have begun to yield promising results.

Qualitatively, there are two methods for the identification of inorganic substances in ashed material. The first, which has been mentioned already, deals with the visual appearances of the ash residues as seen by direct and dark field illumination. The second involves the application of the magnetic electron microscope, which has recently been modified for the special purpose of examining incinerated preparations. The latter method, which obviously provides a more critical means of analysis, remains still in the experimental phase.

a. Optical Differentiation.

The optical method of identifying the inorganic constituents of incinerated sections, according to their visual appearances when observed by dark field, polarized, and ultraviolet light, should be used with caution. Of those substances present in the ash in the form of oxides, only calcium, iron, and silica can be recognized with any degree of certainty. Calcium and magnesium compounds give a white amorphous ash and there are no means of differentiating one from the other by their optical appearances in dark field. The presence of calcium oxide can be detected by the gypsum reaction previously described by Moreau⁵¹. Silica is present as a silico-calcareous compound. It can be recognized by its crystalline appearance and by double refraction when viewed by polarized light. The colour of iron oxide is sufficiently intense in some incinerated sections to give the ash, in which it is mixed, a yellow or reddish tinge. Deep red coloration, according to Policard⁵⁸, indicates the presence of free iron. Before the presence of iron can be assumed with any degree of accuracy it is essential first to examine the preparation by transmitted light, since any carbon residues may yield colour values similar to those of ferric oxide in dark field illumination.

Policard and Okkels⁶⁵ have claimed to detect the presence of uranium salts in incinerated sections, from animals previously poisoned by

uranium, by means of their fluorescence in ultraviolet light. Since extremely small amounts of impurities other than uranium often give a fluorescence, the identification of uranium by visual appearances is open to error. Policard and Pillet⁶⁷ have attempted to identify the chlorides of sodium and potassium in tissue sections by first converting these compounds into sulphates before incineration. They exposed sections to the action of sulphuric anhydride with the result that the sulphates formed in the tissues could be identified in the ash after micro-incineration. In the case of lead, introduced into tissues and organs of experimental animals, Okkels⁵³ has used hydrogen sulphide to form lead sulphide from the ash residues. The black particles of lead sulphide are visible by transmitted light, but it should be noted that the identification of this substance can easily be confused with carbon deposits remaining in the preparation as the result of incomplete incineration.

b. The Electron Microscope.

Bush^{9,10} first described the principles of the electron microscope, which was subsequently modified for use in biological research. The contributions of the electron microscope to medical research have been reviewed recently by Burton, Hillier, and Prebus⁸. The most important technical improvements were achieved by Ruska⁷⁸ in 1933, the power of magnification being increased to 12,000 diameters by the use of two magnetic 'lenses' (iron-encased magnetic spools). In 1935 Martin, Parnum, and Speak⁴⁸ modified the electron microscope for biological research by constructing an instrument which permitted the object to be viewed simultaneously with an ordinary microscope. The magnifications obtained by this instrument were somewhat lower than those obtained by Ruska. It is now possible with the more recent models, which are still in the experimental phase, to examine biological material at a magnification of 35,000 diameters⁸.

McMillen and Scott⁴⁷ in 1937 succeeded in designing an electron microscope capable of being adapted for the identification of certain mineral elements present in ashed tissue sections. The lens of their instrument was a short iron-encased spool similar to that described by Kroll⁴⁰. A further modification was described by Scott and Packer⁹⁶ two years later.

When metals or metallic compounds are heated *in vacuo*, streams of electrons are emitted. The electron microscope makes use of such a beam of electrons in an evacuated tube instead of light rays, as in the case of the optical microscope. By employing suitable magnetic fields placed at different points along the length of the evacuated tube, it is possible to focus the electron beam in such a way that an image is produced on a fluorescent screen at the end of the tube. The

magnetic fields act as 'lenses' and, in the same way that the focal length of an optical system depends upon the wave-length of light, so does the focal length of a magnetic lens vary with the wave-length of the electrons. In order to obtain a magnified image of a tissue section by the electron microscope, the electron beam can be made to originate from the minerals of the actual section itself, or electrons from another source can be directed through a tissue section before coming under the influence of the magnetic lenses to fall finally on the fluorescent screen. The number of electrons emitted by minerals under these conditions varies with the temperature and the type of compound. Hence it is possible to distinguish inorganic substances by their differential emission of thermally excited electrons.

Scott and Packer⁹⁵ in preliminary tests showed that they could differentiate between the different emissions produced in a complex mixture of metallic salts, such as exists in ashed sections. Minute amounts of metallic salts which were placed on the cathode could be readily identified on the fluorescent screen. Numerous similar experiments were repeated under a variety of conditions, after which it was found that all salts present in the test samples, with the exception of iron, produced a recognizable emission. The most brilliant images on the fluorescent screen were obtained from calcium and magnesium. Tests were likewise carried out which more nearly approached the conditions existing in tissue sections. A solution of ashless gelatine impregnated in separate areas by saturated solutions containing calcium, magnesium, iron, sodium, and potassium chlorides was hardened in 10 per cent. formalin prior to dehydration in alcohol and imbedding in paraffin. Sections of 10 μ were cut and immediately placed on a coated cathode. After heating slowly for a period of 90 minutes, the cathode was raised to a temperature sufficiently high for the emission of electrons. With the exception of iron all the chlorides could be identified on the fluorescent screen, the brightest image again being obtained in the case of calcium and magnesium.

Before proceeding to identify mineral substances in tissues by the electron microscope, Scott and Packer⁹⁵ treated all material by the modified Altmann-Gersh frozen dehydration technique. Sections from the frozen-dried material were cut at 10 μ and placed on a nickel cathode which had first been coated with a mixture of 40 per cent. barium and 60 per cent. strontium carbonates carried in a 2 per cent. solution of nitrocellulose in amyl acetate. The cathode coating was allowed to dry before the tissue sections were attached. This coating brings about an 'activation' phenomenon whereby sufficient electrons are emitted to give adequate contrast in the image. After attaching the section by flattening it on the surface of the coated cathode, the vacuum pumps were set in motion and, when a suitable vacuum had been obtained, the

filament current was switched on to heat up the cathode and bring about a gradual incineration of the section. The heating period lasted for about an hour⁴⁷. Following successful incineration, the high voltage and lens currents were then switched on and the electron image of the object was produced on the fluorescent screen.

By this ingenious technique Scott and Packer have so far been able to obtain emission pictures showing remarkable structural and cellular detail, due to the presence of magnesium and calcium in the ashed preparations. These authors state that, by a further modification of the instrument, they should be able to localize sodium and potassium, which at present are both lost from the tissue section during the initial heating and before the electron image is obtained. When further technical modifications of this instrument permit the identification of other mineral elements, a great advance in histochemistry will have been achieved. In fact the successful adaptation of the electron microscope to a mineral study of biological tissues opens up an almost unlimited field for future cytological research. The relative qualitative changes of the inorganic constituents during cellular differentiation in the developing organism, some details of which have already been indicated by ordinary micro-incineration methods, are lines of investigation to which this new technique might be applied with important results. Likewise the study of the changes in mineral substances associated with various diseases opens up a new field for the experimental pathologist. The determination of qualitative mineral changes *in situ*, within individual cells, would give the cytologist an insight into the role which the inorganic constituents play in health and disease.

c. Spectrographic Analysis.

Histo-spectrography, by means of which small areas of tissues are subjected to spectrographic analysis, has been developed by Policard and Morel⁶⁴, Gerlach and Gerlach²², Benoit⁶, and others. This technique has been used in conjunction with micro-incineration for ascertaining the mineral elements present in microscopic areas of incinerated tissues. By passing a high-frequency spark through a selected area of tissue, and analysing the rays emitted with a quartz spectrograph, a spectrogram is obtained which reveals lines characteristic of the mineral elements volatilized by the spark as it passes through a selected area of the tissue section. Scott and Williams⁹⁹, using a Gaertner 1,250 W quartz spectrograph, have obtained strong lines on their spectrogram of Ca, Mg, K, Na, Cu, and P, in a variety of tissues examined.

There are certain technical difficulties in the use of this method as a means of analysing ash residues. In the first place, it cannot be employed for detecting the intracellular distribution of minerals. The burning produced by the high-frequency spark extends over an area of

tissue consisting of many hundreds of cells. Unfortunately, as Scott⁸⁹ has shown, areas of tissue smaller than 1 mm. in diameter do not contain sufficient salts to register a recordable emission spectrogram. Furthermore, if this method is to be employed with micro-incineration, the selection of electrodes must be undertaken with caution, since certain elements of the electrodes are likely to appear in the spectrum. Scott and Williams⁹⁸ have eliminated these difficulties and they have simplified the technique of spark spectrography by screening the electrodes and arranging that the material to be analysed is burnt in the centre of the spark. Yogoda¹⁰⁶ has described a modified technique adapted for examining plant material. Spectrographic analysis at present is useful in a limited way for the estimation of elements in ashed material, but since the results obtained cannot be referred to cellular structures, the method achieves little more than the cruder methods of the analysis of tissues in bulk.

d. Photo-electric Method.

The accurate estimation of the amounts of mineral residues in incinerated cells provides yet another problem of the technique of micro-incineration. A rough idea of the comparative amounts of ash in cells and tissues can be obtained by the visual inspection of the relative densities of the ash in dark field illumination. A finer visual analysis is impossible. In the first place, the quantitative determination of the ash is dependent on the relative thickness of the incinerated sections. Variations in thickness are difficult to avoid even with the best microtomes.

Schultz-Brauns⁸² has devised a technique for the quantitative photography of incinerated sections based upon the standardized development of exposed photographic plates. This method has met with little success. Scott⁹⁰ has attempted to measure the intensity of light reflected from the mineral particles in ashed preparations assuming that the reflected light was proportional to the amounts of ash in different areas of an incinerated section. Preliminary experiments with colloidal solutions indicated that, with a constant source of illumination, the intensity of light reflected from particles in the preparation was proportional to their number. Similar experiments with incinerated material showed that the same principles were involved. With the aid of a specially designed photomicrographic apparatus, a small area of a microscopic field under dark ground illumination is selected and focused by a plano-convex lens on the sensitive plate of a photo-electric cell. The current generated, when the light from the microscope strikes the photo-electric cell, is passed through an amplifier and its output measured by a galvanometer. The amount of light reflected in dark field is so small that the current generated in the photo-electric

cell has to be amplified 40,000 times before a reading on a sensitive galvanometer can be recorded. Although this method does not give an analysis in terms of the weight of ash from individual cells, it does permit the mineral residues of a selected group of cells to be compared in mass with those of another group or layer. These quantitative variations of the inorganic residues in normal and pathological cells of the same type are sometimes important to the pathologist. The investigation of pathological calcifications and other allied phenomena by a quantitative micro-incineration method has given some very interesting data which will be discussed in a later section of this review.

Finally, the important question arises as to whether the distribution of ash obtained in incinerated sections is comparable to that existing in the living tissues and cells. The possibility of a shift of the inorganic salts occurring during fixation and incineration must be considered. In this connexion Scott⁸⁸ has demonstrated that photomicrographs of living cells taken with ultraviolet light of a wave-length of 2,750 Å show an absorption of the rays in precisely those areas of a section which yield ash residues after incineration. One is impressed, moreover, with the remarkably delicate orientation of ash residues resulting from the incineration of secretory granules within the cytoplasm of glandular cells, of chromosomes during cell division and of structures like cilia. It has been noted, for instance, in adipose tissue that the spaces occupied by fats are devoid of ash in incinerated sections, whereas the cell membrane surrounding these spaces yields an ash which corresponds exactly in position and thickness to the membrane observed in stained control preparations. From these and other observations it is safe to assume that the elimination of the organic substances by micro-incineration leaves the inorganic residues in much the same topographical position within a section as exists in the living tissues.

iv. NORMAL CYTOLOGY

a. Blood.

The precision of the micro-incineration technique is fully demonstrated when incinerated films or sections of blood or protozoa are examined by dark field illumination (see Pl. 2, Figs. 1 and 2). The mineral residue of a red blood corpuscle appears to consist of a combination of potassium and sodium with enough iron oxide to give the ash a slight yellow colour. The more complex white corpuscles of the blood, such as lymphocytes and eosinophil leucocytes, leave a more organized ash. They can be readily identified by their inorganic remains. The polymorphonuclear leucocytes, as Scott⁹¹ has demonstrated, possess cell membranes which are clearly defined by an orientation of finely divided ash. There are mineral-free areas in their cytoplasm. Scott has

even been able to detect the mineral residues of burnt blood-platelets which are identified without difficulty, since the blood-plasma leaves no detectable ash.

b. Cell Division.

The behaviour of the inorganic salts during mitosis in tissue cells is an interesting phenomenon which was first described by Scott⁸⁸. The ash from the resting nucleus shows less iron than one would expect and, according to Scott, the bulk of the nuclear ash consists of slightly water-soluble salts which are probably calcium and magnesium. The nucleolus in the resting cell is inorganically differentiated from the remainder of the nuclear chromatin. Most nucleoli contain appreciable amounts of birefringent ash with iron oxide. Both the nuclear and cell membranes in healthy normal cells show a delicate orientation of mineral ash. The cytoplasmic ash in most cells, with the exception of neurones, is thinly and evenly dispersed. Neither the mitochondria nor the Golgi apparatus can be distinguished within the general cytoplasmic ash. At the commencement of mitosis, the inorganic salts of the whole cell appear to become concentrated in the chromatin prior to chromosome formation. This phenomenon is most noticeable in the late prophase, and the fully formed chromosomes of the metaphase consist of aggregations of almost solid white ash. These details are best illustrated during embryonic development in the chick.

In the majority of cells there is a marked tendency towards a concentration of mineral substances along free surfaces, as, for example, in the free borders of the stomach epithelial cells and also in urinary epithelium. This phenomenon^{95,96} is even more marked in the case of the striated border of intestinal epithelial cells previously fixed by the alcohol-formalin method. Such an accumulation of inorganic salts has been reported in the free surface of absorptive epithelial cells by Macklin and Macklin⁴⁶ and by Scott⁹¹. Later Scott and Packer⁹⁵ confirmed these findings and analysed the salts in these regions by the application of the electron microscope. The ash concentrations were found to consist mainly of magnesium and calcium.

Space will not permit a discussion of the general distribution of mineral substances in normal tissues, nor is there any need, as Scott⁹¹ has most ably dealt with these problems in a previous communication. Before passing on, however, a brief reference will be made to several interesting cytological observations recorded in this field. It was always problematical as to whether the pigment in the Ciaccio cells of the mammalian pineal body contained appreciable quantities of iron. Baginski⁴, from incinerated sections of the pineal, was able to determine that the Ciaccio cells contained appreciable quantities of iron. Funaoka and Ogata²⁰ recorded some interesting observations on the nuclear ash

content in the cells of the ovarian tube in *Ascaris megalocephala* and also in the stamens of *Vicia faba*. Kooyman³⁹ has studied in great detail the mineral distribution in the cells of the skin. Also Policard, Noël, and Pillet⁶⁸ described changes in the mineral structure of organs produced by experimental metabolic changes.

c. *Protozoa*.

Micro-incineration of various types of protozoa has added interesting data to our knowledge of these organisms. Differences between the mechanisms of nutrition in various species of infusoria appear to be related to the concentration and distribution of their ash residues. Policard⁵⁹ in 1929 was the first to examine the mineral 'skeleton' of a protozoan cell by micro-incineration. He selected for this purpose the vorticellid *Carchesium polypinum* and described the ashed remains of the vegetative structures as distinct clumps of ash 3 to 4 μ in diameter. The ash of these bodies was whitish in appearance and almost crystalline in structure. The ash of the endosarc contained appreciable amounts of iron, while the myonemata and the basal granules yielded finely orientated white ash residues.

Scott and Horning⁹³ described the ash residues of the binucleate infusorian *Opalina*. They were able to elucidate the cytoplasmic nature of the so-called vegetative bodies which had previously been the subject of much controversy (see Pl. 2, Figs. 1 and 2). Several protozoologists^{28,38,100} had contended that these cytoplasmic structures, as previously identified by somewhat capricious cytological techniques, represented Golgi substance. Incinerated preparations of the organism showed that these vegetative bodies did not consist of Golgi material, since they left a distinctive and organized clump of calcium containing ash. Bearing in mind the method of feeding of this astomatous ciliate and the theory that mitochondria are associated with enzyme activity, it was suggested by Richardson and Horning⁷⁷ that these associated vegetative granules are synthesized under the influence of the mitochondria. Later Horning and Scott³⁷, in a more detailed and comparative study of the differences in the ash organization between a saprozoic and a holozoic infusorian, were able to demonstrate that the mineral distribution in the two types of protozoa could be correlated with their different mechanisms of nutrition. The two types of infusorian selected were *Nyctotherus cordiformis* and binucleate Opalinids infecting *Rana pipiens*.

Since Opalinids are saprozoic and astomatous, the concentrations of inorganic material seen occasionally within the surface cytoplasm may possibly be associated with the mechanism of nutrition. The nature of the mineral salts aggregated in the surface cytoplasm varies considerably in Opalinids infecting different hosts. In some species this ash residue appears to contain large traces of iron and calcium, while in

others there is very little ash in the peripheral areas. At times the concentration of inorganic salts in Opalinids entirely masks such structures as the myonemata and basal granules which are usually visible in incinerated starving Opalinids. Detailed comparative examination of this region of the cell shows not only quantitative, but also qualitative, variations in the inorganic salts, suggesting that this phenomenon is in some manner correlated with the diffusion of digested or digestible material through the cuticle into the protoplasm of the organism. This is of interest in view of the findings of Macklin and Macklin⁴⁶ and Scott and Packer⁹⁵, who showed that aggregations of inorganic salts occur in the free margins of absorbing intestinal epithelial cells.

When the ash residues of the protoplasm and its various inclusions in *Nyctotherus* and *Opalina* are compared, interesting differences are revealed between the general organization of mineral substances composing the skeleton of the holozoic and saprozoic forms. Whereas in *Opalina* the cytoplasm is relatively free from ash, except for the presence of the inorganic remains of the vegetative granules, the general cytoplasm of *Nyctotherus* yields a fine network of mineral residues. Another interesting characteristic of incinerated sections of *Nyctotherus* is the vacuolated appearance of the cytoplasm. These vacuoles are clearly outlined by a deposit of concentrated ash, and they are similar to the vacuoles in the endoplasm of *Vorticella* described by Policard⁵⁹. The nature of the inorganic accumulations forming the vacuolar wall is of considerable interest and importance, since they are relatively rich in iron. The presence of iron at the periphery of these vacuoles might indicate an increased rate of oxidation in these areas. It has been suggested that phosphatides aggregate at the interface of the cytoplasm owing to their capacity to reduce surface tension according to the Gibbs-Thomson law⁴⁹, and possibly they do so in combination with inorganic substances like iron.

Unlike the nuclei in *Opalina* which are characterized by little or no mineral residues, the macronucleus of *Nyctotherus cordiformis* contains so much mineral material that it crumbles when sectioned and cannot therefore be differentiated from the micronucleus in ash preparations. Other structures of these types of ciliates, readily revealed by micro-incineration, are the basal granules, the cilia and myonemata. The clarity with which the ash of delicate structures such as these is shown by the technique, together with general differences in organization and orientation of the inorganic material, opens the way for further studies on these infusorians.

d. Multinucleated Giant Cells.

Okkels⁵⁴ produced multinucleated giant cells experimentally in the peritoneum of the small intestines of mice and examined them in in-

cinerated sections. He found that the ash of the Langhans giant cells was relatively insignificant. The central region of the cell did not contain more calcium than the peripheral cytoplasmic zone. This was a significant observation, being contrary to the general idea that necrotic changes are always taking place in the centre of the Langhans cells.

In cases of inflammatory processes occurring in various non-infective conditions, Okkels found that giant cells were also poor in ash residues. On the other hand, it was interesting to note that giant cells found in relation with human carcinoma of the breast always contained calcium ash. It was further observed that the macrophages containing blood-pigment in inflammatory areas were extremely rich in iron-containing ash.

e. Tissue Cultures.

By means of a slight modification in technique, Horning and Scott³⁵ succeeded in incinerating tissue cultures. Tissues from 7 days' chick embryos were selected for these experiments. Perhaps the most interesting observation recorded was that all cells in the cultures, especially clasmatocytes, which generally contain traces of iron in tissue sections, showed no such traces when ashed in cultures. This was possibly due to the fact that the tissues had been cultivated *in vitro* in a medium entirely cut off from a blood-supply. 'Pure' cultures of epithelium and of fibroblasts were cultivated separately and compared after incineration. The heart fibroblasts contained considerably greater amounts of clumped calcium ash than the cells forming the sheet-like cultures of epithelium. In the latter the cytoplasmic ash was more evenly dispersed, except in the mobilized cells at the periphery of the culture medium.

f. Nerve Cells.

Alexander¹ has examined the mineral contents of neurones in human material. The cytoplasm of ganglion cells in the cerebral cortex was found to be rich in mineral substances which varied in distribution in different parts of the cell. The dendrites and the nucleoli left a distinctive ash, but the remainder of the nucleus was ash-free. The Nissl bodies were found to contain a concentration of calcium. In the general cytoplasm traces of iron and calcium could be identified. The neurofibrillae, as might be expected, were entirely free of mineral residues, and the axon hillock and axis cylinder contained very small amounts of ash which did not appear to consist either of calcium, iron, or silica. The myelin sheaths left no residues at all. It was further noted that the ash of neurones examined during embryonic development was greater in concentration than after differentiation had been completed.

g. Smooth and Striated Muscle.

Scott⁹² has studied the mineral content of muscle by means of incineration as well as by the electron microscope. In smooth muscle the nuclear membranes and the myofibrils yield a distinctive ash which shows a tendency to aggregate at the cell surfaces. The myofibrils retain their individuality in the ash up to the very edge of the fibres. 'Contracture waves' crossing the fibres were inorganically represented by marked concentrations of ash of a distinct bluish-white colour, which differs from that seen in the uncontracted portions of the fibres.

In striated muscle the ash forming the striations has been found to contain iron. Alternating with the striations were areas which were optically mineral-free. The more concentrated ash deposits corresponded with the anisotropic disks, and in all probability the ash-free areas indicated the position of the J or isotropic disks. In well incinerated preparations even the Z band of the J disk and that of the Q disk could be detected. In striated muscle the sarcolemma left a white ash containing calcium.

With the aid of the electron microscope, Scott and Packer⁹⁷ made a study of the location of calcium and magnesium both in smooth and striated muscle. Strips of smooth muscle were taken from the wall of the duodenum of cats and frogs. The pieces of muscle were frozen in liquid air while actively contracting and then dehydrated *in vacuo* before infiltration with water-free paraffin, according to the method already described. This treatment of the muscle precluded any shift in mineral salts within the tissues. In no instance was either calcium or magnesium found to be located outside the cell membranes. The muscle fibres from the frog showed a marked concentration of salts within the sarcoplasm and some indication of the protoplasmic bridges which can be demonstrated in routine stained sections to cross the intervals between adjacent fibres. Otherwise no calcium or magnesium salts could be demonstrated in the endomysium. Scott⁹² has pointed out that these observations with the electron microscope are of interest in view of the recent work of Steinbach¹⁰¹, who contended that calcium is always present in cells in lower concentration than in the surrounding medium.

h. Mammary Gland.

The effects of radium radiations upon the mammary epithelial cells of the mouse, during di-oestrus, oestrus, pregnancy, and lactation, were examined by Horning³² with the micro-incineration technique. No specific variations were found in the distribution of the inorganic constituents of the unirradiated mammary glands fixed during di-oestrus and oestrus. During di-oestrus the sparse secretion often seen

in the ducts in histological preparations was found to leave no ash after incineration. During early and late gestation differences between the amounts of ash in the alveolar epithelium were more marked. Taking into account the morphological fluctuations of the mammary gland cells during pregnancy, the variations in the cytoplasmic ash could be correlated with the degree of cellular differentiation and secretory activity.

White calcium containing ash of the alveolar cells, in material incinerated at 6 days' gestation, was very diffusely arranged, except in the nuclei and towards the apical cytoplasm where it was deposited in thicker concentrations. Material on the 17th day of gestation gave ash residues indicating a greater concentration of inorganic salts both in the epithelium and in the distended lumina of the alveoli. The epithelial cytoplasm presented a honeycombed appearance owing to the complete incineration of the fat droplets which left no inorganic residue. Dense accumulations of calcium were found around the periphery of the spaces once occupied by the fat secretion in the cytoplasm. The white calcium ash derived from the incinerated milk in the distended lumina was more diffusely deposited than in the epithelial cytoplasm. The micro-incineration technique demonstrated very clearly the fluctuations in the inorganic content of the milk secretion. The maximum ash content was reached from the 3rd to the 6th day of lactation, when the milk in the greatly distended alveoli yielded large concentrations of calcium residues (see Pl. 3, Figs. 1 and 2). Material fixed on the 9th to the 11th day of lactation showed relatively less inorganic salts in the lumina. Owing to excessive concentration of ash within the secretory epithelium during early lactation, especially on the 3rd day, cellular details became more difficult to distinguish. The distended alveoli appeared as irregular white rings of ash with the secretion appearing less homogeneous than that found during gestation, owing to the greater fat content.

Radium treatment did not produce any variations in ash distribution of the duct epithelium during di-oestrus and oestrus. Slight but variable increase in ash could be detected in the hypertrophied cells which had been irradiated on the 10th day of gestation and incinerated 5 days afterwards. More specific effects were obtained by irradiating the mammary gland on the third day of lactation. Material fixed 3 days after irradiation showed a striking increase in the calcium content of the milk secretion as compared with unirradiated material at the same stage of lactation (see Pl. 3, Fig. 2). There was no visible effect upon the ash content of the secretory epithelium. The distended alveoli contained a coarsely granular white ash which, owing to the fact that the fat secretion was inhibited by radium treatment, was more evenly distributed than in the control unirradiated sections of the gland (see Pl. 3, Fig. 1). It was of interest to record that in the inorganic

constituents of milk secretion a marked increase occurs following irradiation, whereas the fat secretion is inhibited.

i. The Formation of the Avian Egg-shell.

In the so-called uterus at the terminal end of the oviduct of birds a remarkable mobilization and secretion of mineral salts occurs at repeated intervals to form the calcified shell of the egg. It has been calculated, in the case of the domestic hen, that approximately 5 grammes of nearly pure calcium carbonate become deposited in the course of the 20 to 24 hours' interval during which the egg remains in the uterus. Sections of the uterine mucosa, taken at stages when no shell is being secreted and when a shell is in process of formation, show conspicuous differences in ash content (see Pl. 4, Figs. 1 and 2). Richardson⁷⁶ has demonstrated that the areas of uterine epithelium actually in contact with a developing egg-shell are much richer in mineral residues than adjacent portions of the same epithelium which are not in actual contact at the time of fixation. Unfortunately, the micro-incineration technique did not reveal the mechanism by which the calcium is deposited into the uterine lumen, despite the fact that secretory granules in the uterine epithelial cells were found to leave a well defined ash residue. Since these studies were made, the modern improvements of freezing-drying fixation and the electron microscope have been combined with micro-incineration and it should be emphasized that there is room for much further work with these techniques in an attempt to explain this extraordinary phenomenon of calcification.

j. Teeth.

The mineral distribution in the developing teeth of man, dogs, cats, and rodents has been studied by Hampp²⁶. The findings in incinerated sections of the dental tissues were checked by spectroscopic analysis. The cells destined to become ameloblasts in the developing enamel organ were found to contain relatively large amounts of calcium and magnesium. During differentiation of the ameloblasts a marked diminution of salts occurred, so that the cytoplasm was found to be practically devoid of calcium and magnesium. This loss was accompanied, however, by an increase in other minerals, among which potassium was identified. In the fully differentiated ameloblast, just prior to the formation of enamel, the cytoplasm once again showed large quantities of calcium and magnesium concentrated within the distal and proximal areas of the cells. The central zone of the ameloblast was found to be relatively free from calcium and magnesium oxides. The mineral fluctuations observed in the differentiation and development of the enamel-producing cells were not observed in the odontoblast layer of the tooth germ. At every stage of their development the odon-

toblasts showed a constant mineral content. These cells were distinctly richer in calcium and magnesium when compared with the mesenchyme cells of the dental papilla. Hampp²⁶ found evidence for the assumption that the more mature the odontoblasts became, the greater was their magnesium and calcium content. He concluded from the evidence of these experiments that calcification takes place in alternating rhythms of high and low concentration and not in the form of a steady accumulation of inorganic salts.

k. Embryonic Tissues.

Policard and Pillet⁶⁶ first applied the micro-incineration technique to a study of the developing organism. Their examination was restricted to the calcareous deposits in the endolymphatic sacs of Anurian larvae. They detected appreciable amounts of calcium in the fluid of the cavities of the intracranial diverticula and the saccus endolymphaticus of the embryo. They contended that these deposits formed a reserve of calcium utilized during the development of the larva. Horning and Scott³⁶ studied the changes in the mineral distribution during certain phases of embryonic development in the chick. Apart from describing the ash organization of the developing organs, they found that embryonic development was expressed inorganically by an increase in certain mineral salts and a decrease in others. It was observed that in the brain the calcium salts were progressively increased, while iron was decreased. In the case of some other organs, during different stages of development, a definite migration of inorganic material had taken place. These results of Horning and Scott seem to indicate that mineral migrations take place during organogenesis as well as later during the specific differentiation of cell types, as, for instance, in the enamel organ. Also Alexander and Myerson³ found that the mineral ash in the human cerebral cortex was more concentrated during embryonic development than after differentiation had been attained. The poor concentration of iron oxide in the brain of the new-born infant was assumed to correspond with the lesser degree of vascularity of the new-born infant's brain. Their spectrographic examinations showed that the grey matter of the human adult brain was richer in iron, calcium, magnesium, and sodium than the same areas in the infant brain. The regions of the white matter were richer, however, only in phosphorus content.

V. PATHOLOGICAL CYTOLOGY

a. Arteriosclerosis.

Arteriosclerosis is a disease which, generally speaking, increases in severity and frequency with advancing age. Since the times of Vesalius, the existence of such lesions has been recorded, yet up to the

present time there is no therapeutic treatment which will retard or cure the disease. Many theories of its pathogenesis have been evolved. Of the numerous papers on arteriosclerosis which have appeared during the last five years, some of the most interesting are those which describe the pathological mineral changes as determined by use of the micro-incineration technique. Not only can the topographical position of the inorganic salts in question be examined but, as Policard⁶¹ in his study of the subject points out, 'micro-incineration permits the investigator to determine accurately the very earliest changes in the mineral constituents at the beginning of arteriosclerosis'. It is likely that the study of the very early lesions by micro-incineration will add considerably to our knowledge of the aetiology of the condition.

The haematoxylin staining of calcium deposits commonly employed by pathologists, according to Cameron¹¹, is non-specific. Hence the micro-incineration technique has certain advantages over routine histological methods for the determination of calcium as well as iron and silica.

The manner in which calcium becomes concentrated within the walls of blood-vessels is a phenomenon not fully understood. It appears to be correlated with physical changes in the elastic tissue as described by Wells^{104,105}. Ribbert⁷⁵ was one of the first to show that perfectly normal arteries contain small quantities of calcareous material. Later Faber¹⁸ found that calcium is chiefly located in the elastic laminae of old arteries and is sometimes concentrated in the internal elastic laminae. Calcium deposition is rarely encountered in the intima or adventitia. Considerable debate exists as to whether the calcifications sometimes seen in normal arteries represent an early phase in the aetiology of arteriosclerosis. Ribbert⁷⁵ contends that such deposits bear no relation to the disease, while Faber¹⁸ regards them as an indication of the early onset of the condition. As most of these earlier observations have been based upon Kossa's method of silver impregnation, it is most likely that some of the controversial issues could be satisfactorily settled by studying the early phases of arteriosclerosis with recent micro-incineration technique. A systematic study of the topographical relationship between the mineral constituents in the arterial walls during the early phases of arteriosclerosis would most likely have a direct bearing upon the problem.

Some of the more important contributions in this field are those of Tschopp¹⁰², Ravault^{72,73}, Policard, Morel, and Ravault⁶⁹, Ham²⁵, and Ku⁴¹, all of whom have employed the micro-incineration technique for studying the pathogenesis of the disease. Ham's work on experimental calcification produced by large doses of irradiated ergosterol is most important, since it has brought to light fundamental changes in calcium deposition.

Tschopp¹⁰² was the first to apply micro-incineration to a study of

human arteriosclerosis in the uterus of a woman of only 46 years. He described the localization of calcium and iron salts in the arterial walls and particularly the presence of large amounts of silica in the umbilical arteries of fetuses. He found most of the ash was located in the media, while the intima and the adventitia contained far less mineral residues. Ravault⁷³ gave the first detailed comparative account of ash distribution in histologically normal and diseased arteries. Calcareous deposits with iron oxide were located amongst the connective tissue fibres and elastic tissues, but very rarely within the muscle fibres themselves. In healthy normal vessels the intima and adventitia yield no ash deposits, while calcium containing ash in very small quantities was localized in the media. In calcareous deposits in the inflammatory foci of endarteritis a shift occurs in mineral salts from the media to the degenerating intima. Zinkant¹⁰⁷ has confirmed these findings of Ravault, in a description of increased ash deposits in the elastica interna of the uterine arteries in women of 50 years. As age advances an accumulation of inorganic salts, which were chiefly calcium, takes place in the media and frequently within the internal elastic membrane. Similarly, Policard, Morel, and Ravault⁶⁹ by using spectrographic analysis in combination with micro-incineration were able to find the same sequence of changes in arteriosclerotic vessels in other parts of the body. Policard has also recommended the chemical analysis of the walls of large arteries about 1 mm. in thickness. By means of careful dissection the intima, media, and adventitia can be isolated and analysed separately by ordinary chemical methods. In view of more recent developments in the technique of micro-incineration, there appears little need to employ this tedious procedure by which the topographical deposition of minerals can hardly be expressed in terms of cellular layers.

Ku⁴¹ has reported a series of interesting observations on coronary sclerosis in a selected group of human cases, ranging from the new-born to 79 years of age. The smallest quantities of ash were found in the arteries of new-born infants and then as age advances, small increases in ash content were noted. This gradual increase in ash content was regarded as a normal phenomenon and not necessarily an early indication of the onset of arteriosclerosis. The ashed remains of the coronary arteries were found to consist almost entirely of calcium salts which were localized mainly within the elastic tissue. Much smaller quantities were detected in the other tissues of the intima, media, and adventitia. The manner in which calcium salts are bound to elastic fibres was not determined by Ku. This author also found that as arteriosclerosis develops there is an increase in the amount of elastic tissue which is always accompanied by a corresponding increase in calcium ash. He concluded by suggesting that the increase of the elastic tissue has a direct bearing upon the calcification which occurs subsequently during

the development of the disease. Ku also reported upon the variable increases of mineral constituents found in rheumatic fever nodules and in leucaemic infiltration.

Ham²⁵ in a study of experimental calcification employed the micro-incineration method with great advantage in order to observe rapid changes in calcium deposition following treatment with irradiated ergosterol. It was found that large single doses of this substance produced massive calcification in the aorta, coronary vessels, and cardiac musculature of rats as soon as 48 hours following administration. Incinerated sections of normal aorta from the rat and from material taken 24 hours after the administration of a single dose of irradiated ergosterol showed that the elastic fibres were devoid of mineral residues. In fact detailed examination of sections 24 hours after treatment with ergosterol gave no indication of the high degree of calcification which was about to take place during the following 24 hours. The marked increase in the mineral content of the lesions produced 48 hours after a single dose of activated ergosterol were remarkably well shown in the incinerated remains of sections through the walls of the aorta and coronary vessels. The arteries stood out as thick-walled tubes of mineral residues when compared with the incinerated sections of similar vessels taken from material 24 hours earlier. At 48 hours after treatment the elastic fibres clearly became involved in the process, since they were heavily infiltrated with calcium containing ash. Ham's experiments showed that the calcification was not dependent upon degenerative changes in the tissues affected. Judging from the rapidity and massiveness of these calcifications, he concluded that the phenomenon was related to the inability of the blood plasma to retain all its calcium in solution.

Schultz-Brauns⁸² and Schultz-Brauns and Schoenholz⁸³ consider abnormal deposits of calcium in cells to be due to a decreased functional metabolism probably associated with cell damage. Wehrefritz¹⁰³ and Schoenig⁸⁰ hold the view that the presence of abnormal amounts of calcium indicates a physiological storage of that substance. Policard, Morel, and Ravault⁸⁹ in their spectroscopic studies of the minerals in blood-vessels found that calcium and magnesium were present in small amounts in both normal and pathological vessels. The normal intima contained more calcium than magnesium and the media was even richer in calcium and contained more magnesium than the intima. During lipid degeneration, according to these authors, the intima shows an increased magnesium content, while in calcified atheromata there is a marked increase in calcium and not in magnesium. It was reported that all pathological mineral deposits of the aorta are calcareous and that these conditions are not accompanied by a corresponding increase in magnesium salts. Unfortunately, these authors fail to mention the relationship between the elastic tissue and calcification.

b. Anthracosis and Silicosis.

These pathological conditions have been investigated by Policard and Doubrow⁶³ and by Policard⁶⁰. For the quantitative estimation of the amounts of silica in silicotic nodules, the technique of micro-incineration has obvious advantages. These authors have noted in addition that the iron content of old haemorrhagic lesions in the lung could be demonstrated in ash preparations. Lecloux⁴² when examining tubercular miners for pulmonary anthracosis found that the dust cells in their sputum contained an excessive amount of mineral particles after incineration.

c. Cerebral Lesions.

Cerebral lesions of various kinds have been examined by the micro-incineration technique by Alexander and Myerson². According to these investigators, the inflammatory lesions of syphilitic encephalitis and dementia paralytica are all characterized by a state of hyper-mineralization. The ash accumulates in the nuclei and particularly in nuclear fragments, as well as in the degenerated remains of the infiltrative cells. The cytoplasm of these cells, especially those of glial origin, is much richer in calcium-containing ash than that of normal cells of similar type, while the infiltrative cells in neurosyphilis are extremely rich in iron oxide. Alexander and Myerson also found that the senile plaques in senile dementia and in Alzheimer's disease yield little or no ash after micro-incineration. A difference was noted in the ash of the neurofibrillar strands in Alzheimer's disease, namely, an abnormal amount of calcium-containing ash which was soluble for the most part in water. Later these authors made an extensive study of the changes in mineral constituents of pathological brain tissue by combining the techniques of spectroscopy and micro-incineration. It was found that oedematous brain tissue was richer than normal brain tissue in both sodium and calcium, and that this increase was relatively greater in the white than in the grey matter. In dementia paralytica there is an increase of all mineral substances which is accompanied by a decrease in the iron content of the total brain tissue. It was assumed that this finding indicated that loss of iron was caused by a decrease in the volume of the vascular bed due to a narrowing and obliteration of cerebral vessels, especially the capillaries. In studying cases of lead encephalitis Alexander and Myerson located excessive amounts of lead which were concentrated in the grey matter rather than in the white matter of the brain.

d. Inclusion Bodies.

Routine techniques have yielded as yet little data concerning the chemical nature of the intracellular 'inclusion bodies' caused by the selective action of certain filterable viruses upon animal tissues.

Although much emphasis has been placed upon their morphology and staining reactions, the examination of their inorganic composition has given some valuable information. Owing to the fact that most intranuclear inclusion bodies can be inorganically differentiated by micro-incineration from the ash of the nucleus, it has been shown conclusively that the inclusion bodies are neither artefacts nor simple pathological derivatives of normal nuclear structures. Scott⁸⁷ was the first to incinerate material containing inclusion bodies, and he found that the large nuclear inclusions caused by the sub-maxillary virus in guinea-pigs were almost ash-free, while the peripheral chromatin and the nucleolus were abundant in mineral substances. Cowdry's observations¹⁵ on the intranuclear inclusions in the liver cells of monkeys infected with yellow fever showed that these structures contained very little ash and that they could be readily differentiated by their mineral organization from both the nucleolus and the chromatin. Covell and Danks¹⁴ reported that the cytoplasmic inclusions of rabies consist of a compact inorganic residue of white calcium ash. The Borrel bodies of fowl-pox were found by Danks¹⁷ to leave an abundant and characteristic mineral residue composed largely of calcium. He concluded that the relatively large deposits of inorganic material in these structures served as a focus for the adsorption of the virus.

Rector and Rector⁷⁴ have demonstrated that the ash of the herpetic intranuclear inclusions in the cerebral cortex of rabbits varies according to the age of the inclusion body, the more mature inclusions containing appreciably less ash residue than those which were immature. In the advanced stages of the disease the inclusion body leaves little or no mineral residue, although the nuclei and chromatin still maintain a constant amount of mineral material, despite the striking changes in the cells resulting from the action of the virus. A similar differentiation between the ash resulting from the incineration of chromatin, nucleoli, and inclusion bodies has been demonstrated by Horning and Findlay³³ in cells infected by the Rift Valley fever virus. The residues forming the marginated chromatin consist mainly of calcium containing ash. Findlay¹⁹ had previously shown that these peripheral nuclear aggregations of chromatin were due to the action of the virus upon the cell. The nucleoplasm was devoid of ash, while the nucleolus formed a more compact mass of inorganic material with a distinct yellow tinge suggesting the presence of iron oxide. The intranuclear inclusion was represented by a finely marginated inorganic residue, which was typical in appearance of the less mature inclusions of *Herpes simplex* described by Rector and Rector. No diminution of ash was observed in the inclusion bodies of Rift Valley fever such as these authors recorded in the herpetic inclusions. Nor was any visible increase in ash content of the inclusion body detected during acute liver necrosis, although this was

shown to be associated with a marked increase and clumping of calcium salts in the cytoplasm of liver cells. The ash distribution resulting from liver necrosis produced by the virus of Rift Valley fever was compared with the ash residue of liver from uninfected animals in which necrosis had been produced experimentally by carbon tetrachloride poisoning, and in malignancy. Horning and Findlay found that the necrosis produced by these agencies, as well as that obtained with several other types of virus infection, led to a marked increase of calcium containing ash in the liver cells followed by a final disappearance of the inorganic residues accompanying cellular degeneration. The final liberation of the inclusion body from the disintegrating nucleus did not influence the incineration picture in the case of material infected with Rift Valley fever. The mineral differentiation between the nucleolus and the inclusion bodies should help to settle the status of those inclusions which, from the evidence of staining reaction, are thought to be of nuclear origin.

e. Neoplastic Growths.

The question as to whether malignancy involves an increase or diminution in the mineral content of tissues was finally decided by the application of the micro-incineration method. The localization and orientation of the inorganic salts in normal and neoplastic cells *in situ* has been investigated by many workers. Policard and Doubrow⁶² first examined tumour tissues by the micro-incineration technique and were impressed by the fact that cancerous tissues remain carbonized longer than normal tissues. Scott and Horning⁶⁴ studied human medullary duct carcinomata of the breast together with several examples of scirrhus types. It was shown that the infiltrating new growths were characterized by a heavy deposition of mineral salts and in comparison with similar normal tissues they were found to contain a relatively greater amount of mineral ash, especially of calcium and iron oxides. The nuclei of the malignant cells were not only richer in ash but contained more iron than the nuclei of normal healthy duct cells. The nuclear ash was concentrated along the nuclear membrane.

Further investigations on the mineral organization of a series of human neoplastic growth have been made by Olch⁵⁵ and by Olch and Scott⁵⁶ in which they likewise described differences in the ash distribution between normal and malignant growths. Human naevi and hyperkeratoses were reported to possess a bluish ash in the areas of epithelial proliferation, which probably indicated an increased content of sodium. These authors made no mention of the presence of iron oxide in the incinerated sections of naevi. Horning and Lamb³⁴ examined human melanomata arising from the skin and from the choroid coat of the eyeball. The pigment of the melanotic sarcomata of the eye yielded an iron-

containing residue, while the numerous pigmented naevi were optically devoid of iron. This was of interest since the opinion is generally held that all melanomata contain variable amounts of free iron pigment. Alexander and Myerson² examined tumours of the brain, gliomatous as well as metastatic varieties, and confirmed the findings of Scott and Horning⁹⁴ especially with regard to the increased nuclear accumulations of iron-containing ash in tumour cells. They also reported that all tumour cells were extremely rich in mineral substances when compared with normal brain cells. It was further found that most of the increased white cytoplasmic ash and the yellow nuclear ash of malignant brain cells were insoluble in water. These authors concluded that the increased mineral content of tumour cells might possibly play a role in X-ray sensitivity. Alexander and Myerson³ in a later communication confirmed certain of their findings by using spectrographic analysis with micro-incineration. They found that the ash of meningiomata was 10 times richer in calcium than the normal grey matter, while other mineral substances were diminished in concentration. The mineral ash of a spongioblastoma of a mixed type, with a good deal of protoplasmic astrocyte differentiation, showed more potassium but less magnesium and phosphorus than normal grey matter.

Micro-incineration studies on tar tumours of rodents have also been described by Horning³⁰. Detailed comparisons were recorded between the ash of the hypertrophied tarred skin in mice and that of the underlying carcinoma cells in their varying degrees of differentiation. Variations in the mineral residues which accompany the skin hyperplasia and the subsequent development of tumour cells were observed. An increase of calcium ash was found consistently in the malignant and also in the hypertrophied stroma cells (see Pl. 5, Figs. 1 and 2). In this respect the tar tumours stand in contrast to those previously recorded in which an increase of ash was found only in the malignant cells. The changes in the inorganic content of the epidermis during the process of keratinization were observed. An increase in calcium ash in the cell nests involved in this process leads to an obliteration of all cellular individuality in the ash. The iron-containing yellow ash of keratin can be traced amongst the flat white calcium ash of the disintegrating necrotic tumour cells. The fully formed keratin pearls consist of a series of concentrically orientated residues of deep yellow ash. A relationship between the ash content of the nuclei of tumour cells and radio-sensitivity has been suggested by Cathie and Davson¹³. They claim to have demonstrated that the degree of radio-sensitivity of tumour cells is dependent upon the amount and arrangement of the nuclear inorganic material within the malignant cells. In support of their contention, they quote the work of Lieber⁴³, who has shown that the more malignant the tumour cell the greater the potassium calcium ratio. The results of Cathie and Davson

show that there are differences in the amounts and distribution of the nuclear ash between the less radio-sensitive squamous-celled carcinomata and the more radio-sensitive basal-celled tumours. These types of tumours were selected because they showed, within reasonable limits, a constant difference of sensitivity to radiation. The radio-sensitive basal-celled epitheliomata were found to possess a larger nuclear ash content than the less radio-sensitive tumour cells. Later Cathie¹² introduced the photo-electric method for obtaining more accurate comparative measurements of nuclear ash differences. Nineteen cases of human new growths of which the radiation lethal dosage was accurately known were selected for these experiments. These results confirmed the former findings of Cathie and Davson that the inorganic nuclear ash could be employed as an index of tumour radio-sensitivity.

The action of radium radiations upon the inorganic structure of tumour cells has been investigated by Horning³¹. Incinerated sections of adenocarcinoma 27, a transplantable mouse tumour, were examined after periods ranging from 4 hours to 24 days following radium application. An increase in white calcium ash was detected as soon as 4 to 6 hours after radiation. This phenomenon was maintained in the cytoplasm 24 hours after radium treatment, and was accompanied by a marked cellular hypertrophy on the 3rd day. The maximum increase of mineral salts occurred on the 6th day following irradiation, at which time degenerative areas were found in all tumour-cell masses. The final disintegration phases of the dying tumour cells were characterized by alterations in the inorganic constituents in the form of a marked increase in calcium-containing ash. When reviewing these results it must be considered whether the increase in mineral salts, so conspicuous in tumour cells as soon as 4 to 5 hours after radium treatment, is due to the immediate action of the rays or to a secondary phenomenon following cell degeneration. In this respect it is interesting to note that Ludford⁴⁵, using a similar radium applicator for the same period, recorded definite cytological changes in the malignant cells of adenocarcinoma 27 after 40 minutes irradiation. It might therefore be concluded that the increase in ash observed after 4 hours irradiation was the result of a direct action upon the cancer cell. The maximum increases in calcium-containing ash, detected 5 to 6 days after radium treatment, was apparently due to a combined effect of the direct action of the rays, together with an indirect effect upon the stroma and tumour parenchyma, previously described by Bashford, Murray, and Cramer⁵ and Cramer¹⁶. The gradual and extensive development of the stroma and the destruction of the blood-vessels influenced by radium treatment would obviously lead to degeneration and necrosis. These aspects of the problem were clearly revealed by the micro-incineration process. The marked increase in calcium ash in necrotic areas of the irradiated

tumours was similar to that previously reported by Scott and Horning⁹⁴ in human duct carcinoma of the breast.

At present it is difficult to explain these findings, especially the increase of calcium ash in tumour cells, following short intervals after radiation, but it seems obvious that any increase in calcium can only come from the blood-stream, in which case the radium radiations might affect the permeability of the cell membranes in such a manner as to allow an increased penetration of calcium ions into the malignant cells.

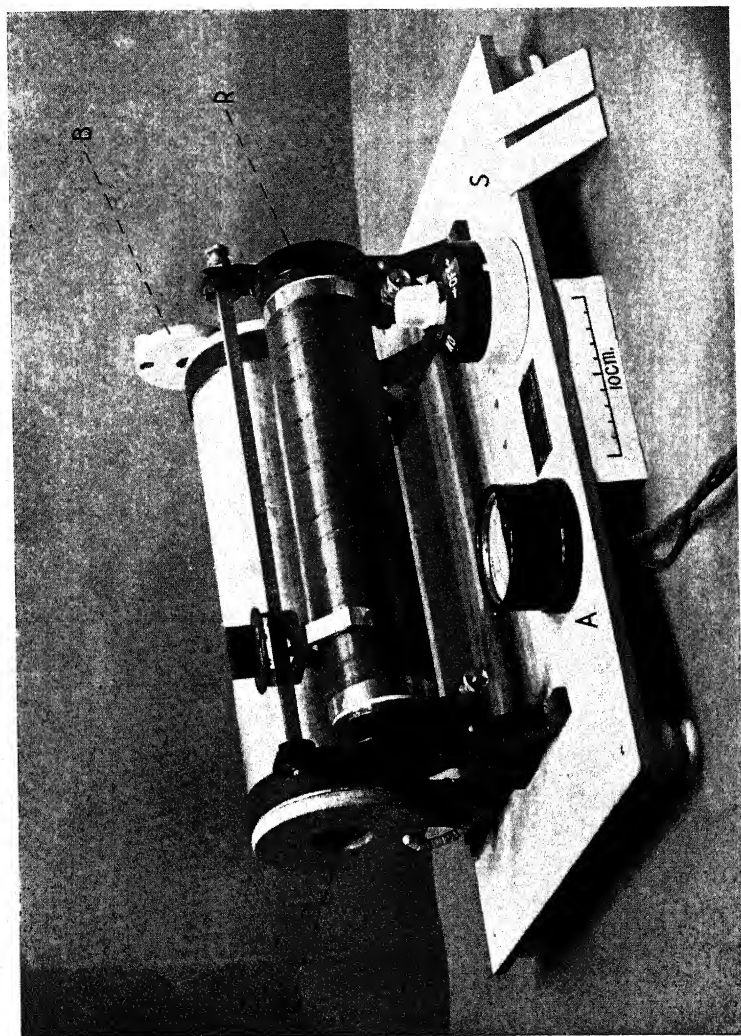


FIG. 1. Photograph of electric quartz incinerator. A=ammeter, B=door, used for regulating current of air, Q=quartz tube surrounded by heating element, S=silica slabs on which the glass slides bearing the sections rest inside the furnace, R=rheostat by which the temperature of the apparatus is controlled.

E. S. Horning



FIG. 1. Photomicrograph of control section, cut through the rectum of *Rana pipiens*, infected with Opalinids and *Nyctotherus cordiformis*. The vegetative granules in the elongated Opalinids and the macronuclei in the *Nyctotherus* are clearly shown.

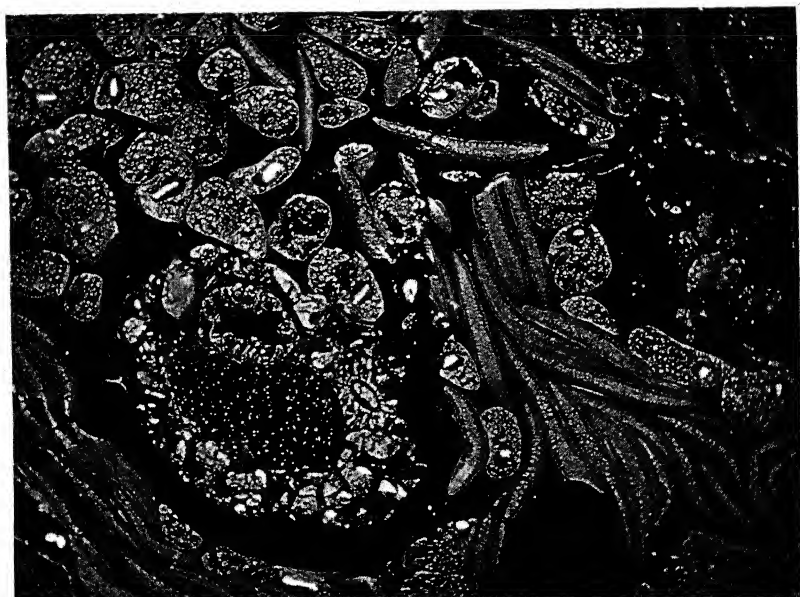


FIG. 2. Incinerated section, similar to the above, photographed by dark-field

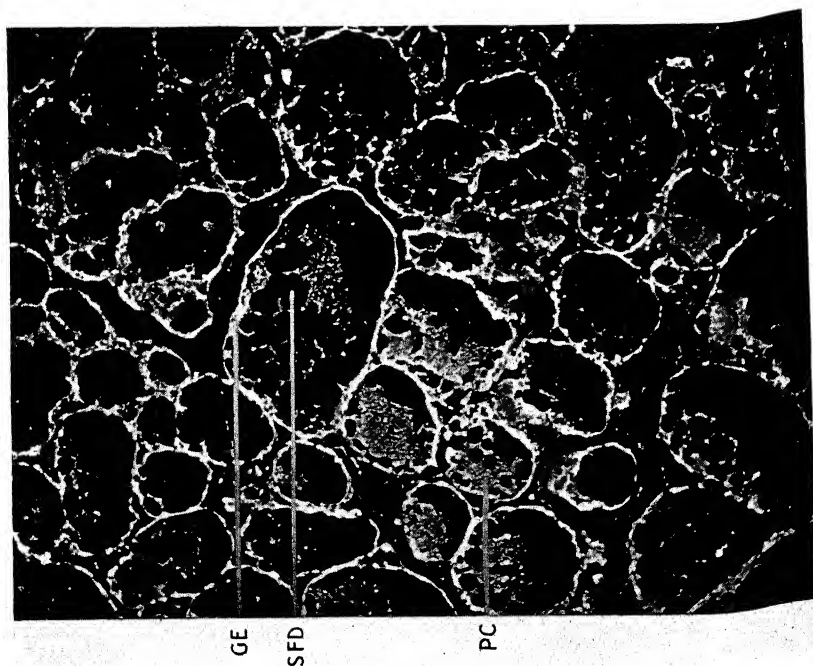


FIG. 1. Photomicrograph of an incinerated section from an unirradiated lactating mammary gland of a mouse (dark-field illumination).

PC=protein coagulum.
GE=glandular epithelial cell.

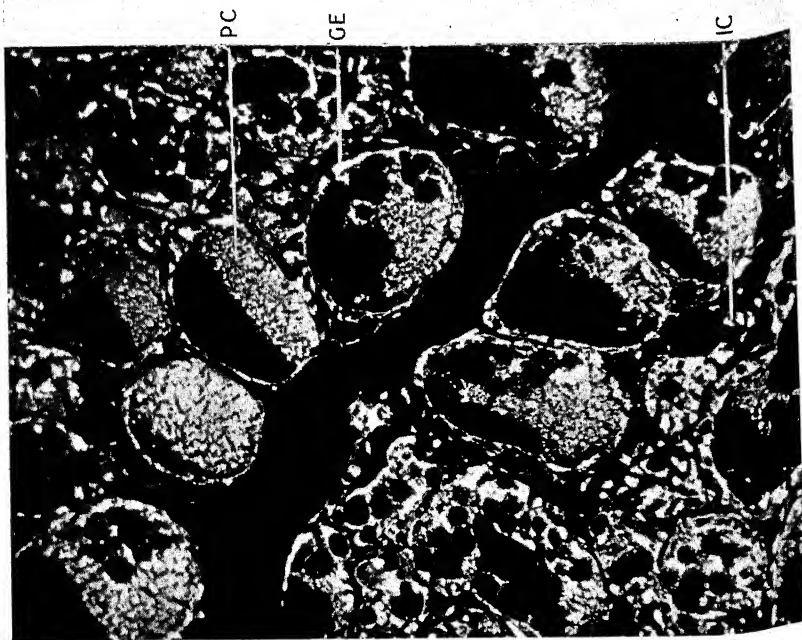


FIG. 2. Photomicrograph of an irradiated lactating mouse mammary gland, taken in dark field after micro-incineration. A marked increase is shown in the morganic salts of the milk, after irradiation.

IC=Intralobular connective tissue.
SFD=Space once occupied by fat.

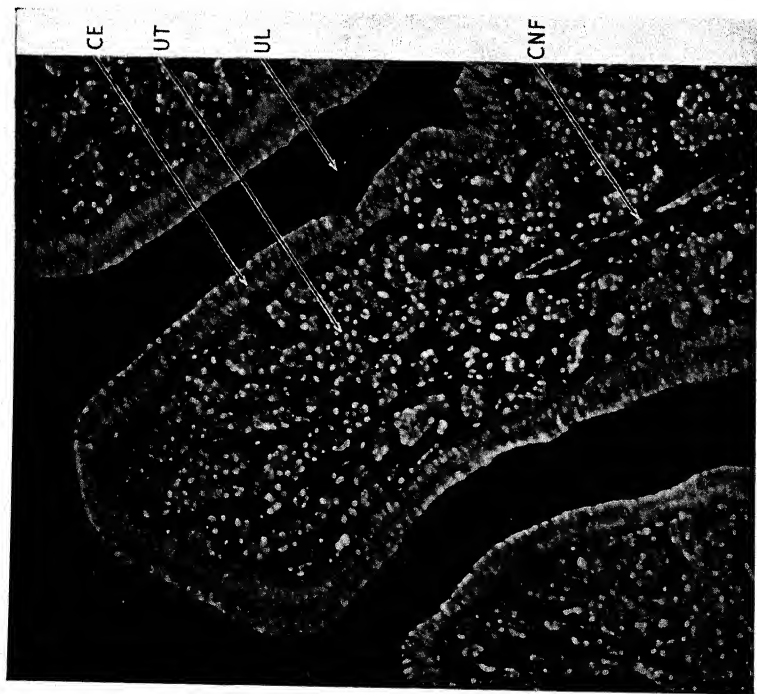


FIG. 1. Portion of the uterine mucosa taken from a domestic hen at a time when the uterus contained a soft, partly calcified egg-shell. There is a heavy concentration of white ash in the incinerated section, which is localized particularly in the surface epithelium. $\times 175$.
CNF = Connective tissue axis of fold. UL = Uterine lumen. UT = Uterine gland.

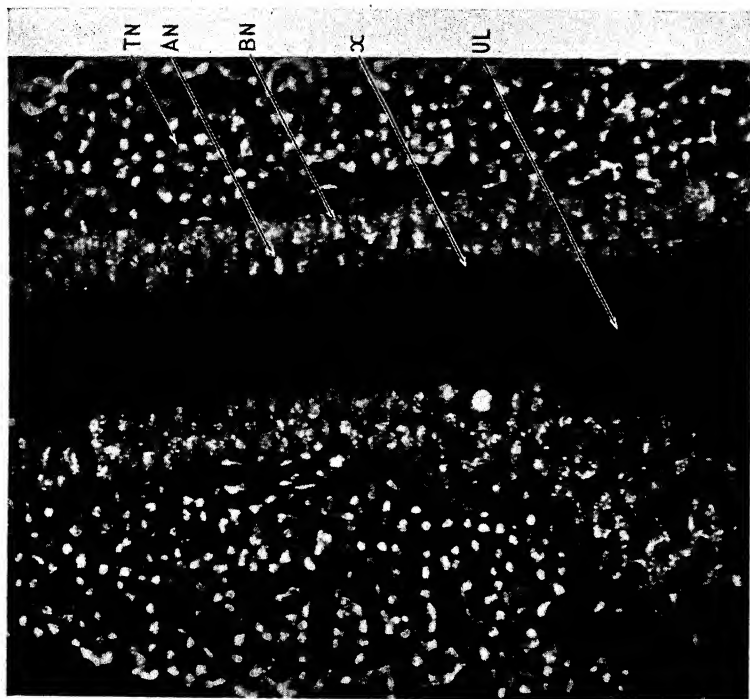


FIG. 2. A similar area of uterus, under higher magnification, from a fowl which had laid an egg about 4 hours previously. The lining epithelium of the empty uterus is conspicuously free from ash residues except for the nuclear zones. $\times 300$.
 α is used by the author (K. G. Richardson) to indicate special points in the figure referred to in his text. UL = Uterine lumen. BN = Basal epithelial nucleus. AN = Apical epithelial nucleus. TN = Gland epithelial nucleus.



FIG. 1. Photomicrograph by direct illumination of a control section of a mouse tar tumour, stained with Ehrlich's haematoxylin, after fixation in alcohol-formalin, showing the tarred skin, the stroma, and the underlying tumour cells.

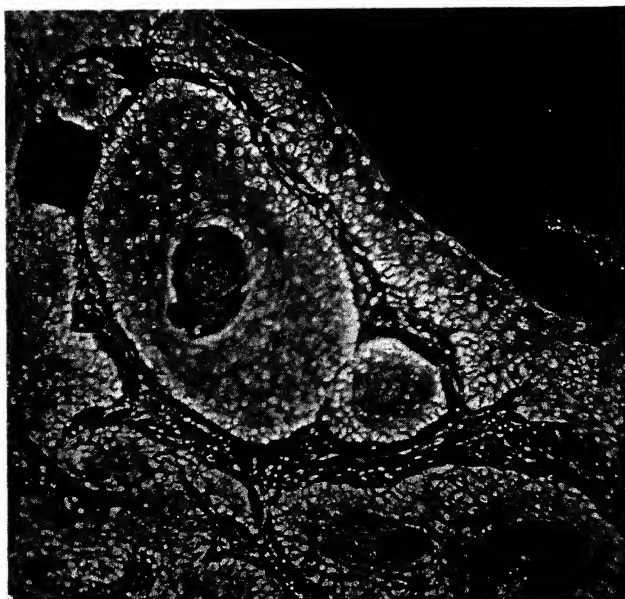
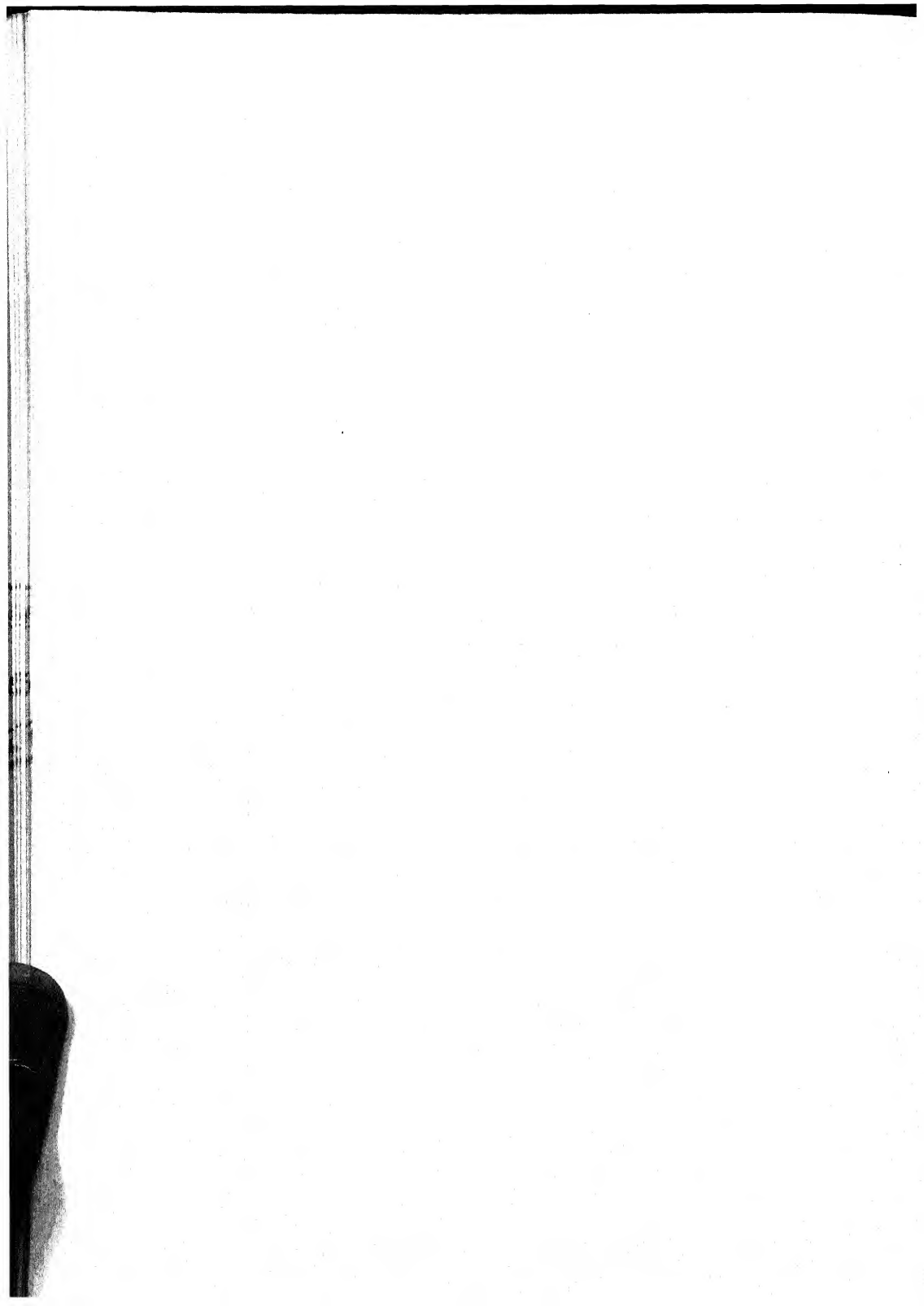


FIG. 2. Incinerated section, similar to above, photographed by dark-field illumination.



CHAPTER VII

ENZYME SYSTEMS OF CELLS

By H. BLASCHKO and W. JACOBSON

i. INTRODUCTION

ALTHOUGH much is known about the occurrence of enzymes in tissues, in body fluids, and in the secretions, precise information as to their distribution within the organs and cells and the site of their formation is scarce. Recent great advances in our knowledge of the chemical nature of enzymes have been achieved by methods which usually involve the destruction of the structural elements of the tissues. The biologist is not satisfied with the statement that a given enzyme occurs in a given organ; he will ask: In which cells does the enzyme occur? where is it localized within the cell? and where is it formed?

Enzymes are found, not only in cells, but also in the body fluids (e.g. in blood-plasma) and in the secretions, but it is likely that they are always formed in cells. Of the intracellular enzymes *sensu strictiori* it was previously believed that their activity was an activity characteristic of the living cell. Pasteur believed that the presence of living cells was an indispensable condition for the occurrence of alcoholic fermentation, and it was a revolutionary discovery when Buchner showed that cell-free extracts were capable of catalysing the fermentation of sugar.

The aim of the chemist is to study the reaction catalysed by an enzyme in a system which contains a purified enzyme preparation, the substrate and, if necessary, coenzymes and inorganic constituents. Such a system is a model of what occurs in the living organism. Attempts to reconstruct the complete system with purified substances have in some instances been successful; in other cases this has hitherto proved impossible. Occasionally the destruction of the tissue abolishes the metabolic activity under examination.

Enzymes may be conveniently classified in three groups:

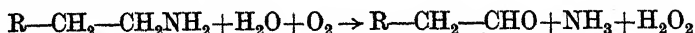
a. *Enzymes which can be obtained in solution.*

This group contains all the extracellular enzymes and a number of 'endo'-enzymes, namely, those which can be eluted from the tissue when the structure is destroyed. Many enzymes of this group have been purified.

b. *Enzymes not separated from the insoluble tissue constituents.*

Enzymes of this group are studied *in vitro* by using tissue mince in fine suspension. Cytochrome oxidase and amine oxidase are repre-

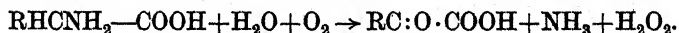
sentatives of this group. The amine oxidase of vertebrate liver which catalyses the reaction:



is always found associated with the granules of the liver cells, but if the granules are destroyed by lysocithine, a powerful cytolytic agent, the activity is not impaired. There exists no sharp boundary between enzymes of this group and the soluble enzymes. Some chemists are inclined to believe that all enzymes of this group may be rendered soluble by suitable methods. While that may be so in some cases, it is quite possible that some enzymes are inseparably attached to the insoluble proteins of the cells.

c. Enzymes which are inactivated by the destruction of the tissue.

The oxidation of fatty acids may serve as an example; this metabolic process can be studied only in the intact animal or in isolated organs. The tissue slice technique is especially useful for this type of reaction. One case which is usually included in this group is especially well studied; it is that of the amino-acid oxidase which catalyses the oxidative deamination of the amino-acids of the naturally occurring *l*-series:



It was originally believed that the enzymic activities of *l*-amino acid oxidase were destroyed by grinding the tissue (e.g. pig's kidney); but it was shown by Krebs⁵³ that the loss of activity was not due to the breaking up of the structure, but to the dilution of the extracts. If the undiluted (or almost undiluted) tissue detritus is examined, it is found that it retains the enzymic activity, but on dilution the activity falls more rapidly than in proportion to the degree of dilution. According to Krebs, this indicates that the reaction catalysed requires the presence of *three* components, viz. substrate, enzyme, and a third factor, e.g. a coenzyme. The chances for a ternary collision must decrease rapidly with dilution. In enzymic systems of this type the spatial relationship of the components in the tissues must be of great importance.

The way in which enzymes are eluted from tissues has been used for a classification of enzymes by Willstaetter and his collaborators. Enzymes are assumed to be bound to the proteins of the protoplasm in 'symplexes'; some of these symplexes are split very easily, in which case the enzyme can be obtained in solution and is called a 'lyo-enzyme'. Other enzymes cannot be separated from the insoluble cell proteins; they are called 'desmo-enzymes'. This classification of enzymes in desmo- and lyo-enzymes has yet to prove its usefulness; the boundary between soluble and non-soluble enzymes is not a sharp one. Moreover, it depends on the method of elution employed. The reader is referred to a review on the subject by Bamann and Salzer². Willstaetter and Rohde-

wald¹⁰¹, in a study of the state of glycogen in cells extend these conceptions to this substance and distinguish between desmo- and lyo-glycogen.

The localization of enzymes within cells must clearly be of great importance for the regulation of enzymic reactions in the cells. This was first clearly recognized by Claude Bernard⁴ when he discovered both glycogen and the diastatic enzyme which hydrolyses glycogen in the liver cells. For instance, the liver of the hibernating frog contains much glycogen and little glucose, that of the summer frog much glucose and little glycogen. The enzyme is always present in the liver; why does it act differently in the two cases? Various reasons have been suggested. According to Lesser, surface-active substances in the cells may affect the accessibility of the substrate for the enzyme. It has recently been suggested by Seckel⁹³ that the bile salts in the liver cell may be of importance in this connexion.

Enzymic reactions and the distribution of enzymes in cells and tissues can be studied by various methods. In a number of cases the enzymic systems can be observed in the tissues, e.g. by spectroscopic methods. Keilin has used these methods successfully in the study of the intracellular oxidation catalysts. In order to obtain information about the localization of enzymes in tissues and cells, the enzymic activities can be measured and compared with the cytological composition. This method involves the elaboration of micro-methods for the determination of enzyme activity; it has been chiefly used by Linderstrøm-Lang and his collaborators. Lastly, there exist enzymic reactions which lead to characteristic reaction products which can be seen in cells. Dopa-oxidase and peroxidase have been studied by this method.

ii. RESPIRATION ENZYMES

Paul Ehrlich was the first to use cytological methods in the study of cell oxidation. In his book *Das Sauerstoffbedürfnis des Organismus* (1885) he attempted to obtain information on the intensity of tissue oxidation in various organs by intravital injection of dyes which are reduced to leuco-derivatives. Ehrlich's work—which laid the foundation stone of the technique of intravital staining—did not lead to an understanding of cellular oxidations; specific affinities of the dyes to the structural elements introduced difficulties which made it impossible to interpret the results obtained. But of the dyes employed by Ehrlich two have proved of great importance in the study of biological oxidations: methylene blue in the work on dehydrogenases and indophenol in the work on the cyanide-sensitive oxidation catalysts. Dehydrogenases and 'indophenol' oxidase (now correctly called cytochrome oxidase) are the two main types of intracellular oxidation catalysts. They are briefly reviewed in the following, in as far as they are accessible to cytological examination.

a. Cytochrome System.

The importance of iron in cellular respiration was first recognized by Warburg¹⁰⁰. His discovery that the chief respiratory catalyst (Warburg's respiratory enzyme*) contained haemin-iron is not based on direct spectroscopic observation but on more indirect evidence: cellular respiration is inhibited by carbon monoxide and the inhibition is reversed by light. Dissociation by light is known to be a property of iron-CO compounds. Warburg determined the relative efficiencies of monochromatic light of different wave-lengths in reversing the carbon monoxide inhibition of cell respiration and showed that the 'efficiency spectrum' thus obtained was that of a haemin compound. In 1925 Keilin⁴⁴ discovered the importance of the intracellular haemin compounds (McMunn's histo- and myohaematin) by direct spectroscopic observation of respiring tissues or cells. These substances, which were shown to be present in oxidized or reduced form, he called cytochromes. They occur in most living cells.

Tissues suitably illuminated show the red colour of cytochrome; this can be seen in a slice of brain tissue carefully washed free of blood, or in a thick suspension of yeast, observed in transmitted light. Suitable materials for the study of the cytochromes in cells are the thoracic muscles of living insects (*Galleria*) or suspensions of baker's yeast. Keilin^{44, 45} showed that the typical cytochrome spectrum was due to the presence of at least three substances which he called cytochromes a, b, and c (see Text-fig. 1). They give typical haemin spectra; the bands of the reduced cytochromes from different sources are shown in Text-fig. 2. A thick suspension of baker's yeast shows the bands of reduced cytochrome which disappear on shaking with air. Cytochrome has been oxidized. If the suspension is left standing, cytochrome is again reduced. The rate of reduction is dependent upon the presence of substrates for oxidation. Reduction occurs rapidly if the cells are saturated with substrates and slowly if they are relatively starved. The oxidation of cytochrome on shaking is not due to an autoxidation: the rate of oxidation is slowed down by cyanide or carbon monoxide, and these substances do not combine with the cytochromes a, b, or c, as there is no change in the spectrum on the addition of CO or KCN. These inhibitors act on an enzyme which oxidizes cytochrome: cytochrome oxidase. Keilin has shown that cytochrome oxidase exhibits all the characteristic properties of Warburg's respiratory enzyme. Moreover, he showed that the enzyme was identical with the long-known indophenol oxidase of mammalian tissue. This enzyme catalyses the oxidation of the 'nadi' reagent (dimethyl-paraphenylenediamine + α -naphthol) to indophenol and that of paraphenylenediamine to a diimine. Keilin and Hartree^{46, 47, 48} have shown that the enzyme does not

* For literature see Reid⁸⁴.

directly oxidize these substances: they are oxidized by oxidized cytochrome. The re-oxidation of the reduced cytochrome by atmospheric oxygen is catalysed by the cytochrome oxidase. Quite a number of substances are oxidized by the cytochrome—cytochrome-oxidase system in a similar manner, e.g. polyphenols (adrenaline), ascorbic acid, sulphhydryl compounds (cysteine). Cytochromes seem to be the only substances which are directly oxidized by the cytochrome oxidase.

	a	b	c	d
Cytochrome	α_1	α_2	α_3	$\beta_1, \beta_2, \beta_3$
Compound a'	α_1			β_1
" b'		α_2		β_2
" c'			α_3	β_3
	Red			Blue

TEXT-FIG. 1. The upper line gives the absorption bands at reduced cytochrome. The three lower lines give the position of the bands of the components a, b, and c. After Keilin⁴⁵.

	610	605	600	595	590	585	580	575	570	565	560	555	550	545	540	535	530	525	520
6032	a	Bacillus subtilis	5660	b									5502	c				5270	d
6035	a	Yeast cells	5645	b									5490	c				5190	d
6035	a	Eschallot bulb	5640	b									5500	c				5190	d
6046	a	Bee wing muscles	5665	b									5502	c				5210	d
6038	a	Dytiscus wing muscles	5664	b									5495	c				5205	d
6046	a	Galleria wing muscles	5657	b									5495	c				5200	d
6035	a	Snail radula	5650	b									5495	c				5200	d
6040	a	Frog heart muscles	5660	b									5500	c				5205	d
6045	a	Guinea-pig heart muscles	5662	b									5500	c				5205	d

TEXT-FIG. 2. Positions of the four absorption bands a, b, c and d, of reduced cytochrome in various organisms. After Keilin⁴⁵.

In addition to the three cytochromes a, b, and c, Keilin and Hartree recently described a new component which they call cytochrome a₃. This substance displays remarkable similarities to cytochrome oxidase. It is not yet known whether this component contains haemin-iron or copper. Apart from component c, the different compounds have not yet been isolated, and their characterization is based on the observation of the absorption spectra. In the following paragraphs a short summary

of the properties of the components, as recently described by Keilin and Hartree, is given:

Component a is a haem-protein compound, insoluble or intimately bound to the insoluble material of the cells. It is thermolabile and does not combine with CO, KCN, or other inhibitors.

Components a₁ and a₂ are found only in a few bacteria devoid of component *a*.

Component a₃ is thermolabile; it reacts with CO and KCN; it is autoxidizable. It shares these properties with the respiratory enzyme, cytochrome oxidase. It is not certain whether component *a₃* is in fact identical with cytochrome oxidase, since (1) the CO compound of *a₃* does not dissociate in light, and (2) the oxidized component *a₃* does not oxidize anaerobically the reduced component *c*.

Component b has also up to now proved insoluble. In contrast to component *a* and *c*, it is somewhat autoxidizable. In the presence of urethane and a suitable substrate, e.g. succinic acid, component *b* remains reduced, whereas components *a*, *a₃*, and *c* are oxidized. There is reason to believe that substances like polyphenols (which react with cytochrome without a specific activator) do not require this component, but that it is required for the oxidation of activated substrate molecules such as succinic acid (see below).

Component c is the only one that is soluble; it has been separated and prepared in a pure condition; it is relatively thermostable. It does not combine with CO or KCN. It is not autoxidizable under physiological conditions.

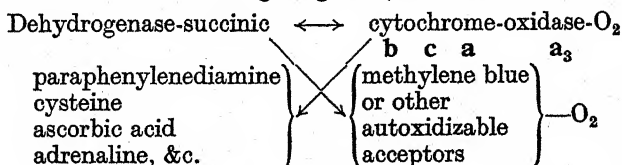
The position of the characteristic absorption bands of the different components are given in the accompanying table (after Keilin and Hartree).

In higher animals the typical cytochrome spectrum as described above is found only in heart and skeletal muscle and in the grey matter of the brain. In kidney, liver, and pancreas Keilin and Hartree find the cytochrome a spectrum rather faint and the bands of *b* and *c* replaced by a broad band which they call *b₁*.

Most of the normal oxidizable substrates of the tissues differ from substances like paraphenylenediamine or adrenaline in that they are not directly oxidized by the cytochrome—cytochrome-oxidase system; they require the presence of a catalyst which has a high degree of substrate specificity. The interrelationships between the cytochrome system and the specific activators of the substrates ('dehydrogenases') have been studied by Keilin in a preparation from mammalian heart muscle which, when complete, contained the following constituents: succinic acid, succinic oxidase ('dehydrogenase'), cytochromes, cytochrome oxidase, oxygen. The properties of the cytochrome system can be studied by replacing the succinic-acid—succinic-oxidase by substances such as paraphenylenediamine; the properties of the dehydrogenase system

Components of cytochrome	Notation of bands		Position in $m\mu$
	Previously used	Proposed now	
<i>a</i>	<i>a</i>	<i>a</i> α	605
	..	<i>a</i> β	?
	..	<i>a</i> γ	452
<i>a</i> ₃	..	<i>a</i> ₃ α	600
	..	<i>a</i> ₃ β	?
	..	<i>a</i> ₃ γ	448
<i>b</i>	<i>b</i>	<i>b</i> α	564
	<i>d</i>	<i>b</i> β	530
	..	<i>b</i> γ	432
<i>c</i>	<i>c</i>	<i>c</i> α	550
	<i>d</i>	<i>c</i> β	521
	..	<i>c</i> γ	415
<i>a</i> ₃ ·CO	..	<i>a</i> ₃ CO α	590
	..	<i>a</i> ₃ CO β	?
	..	<i>a</i> ₃ CO γ	432
<i>a</i> ₃ ·CN	..	<i>a</i> ₃ CN α	590
	..	<i>a</i> ₃ CN β	?
	..	<i>a</i> ₃ CN γ	450

can be studied separately by replacing the cytochrome system by autoxidizable substances such as methylene blue. These interrelationships are shown in the following diagram (after Keilin and Hartree):



Attempts to separate components of the dehydrogenase-cytochrome system (apart from the soluble component *c*) have been unsuccessful. The activity of the preparations from heart muscle seems to depend on the way in which the single components are built into the framework of the insoluble tissue proteins.

b. Dehydrogenases.

The system which activates succinic acid, the succinic dehydrogenase, has not yet been isolated. But a number of dehydrogenases have been isolated and partly characterized chemically. Usually these enzymes are specific proteins which act in conjunction with substances which can be split off from the protein—sometimes with great ease, sometimes only with difficulty. These substances are reversibly oxidizable and act in the oxidized condition by oxidizing the substrate molecule. These

prosthetic groups have been seen in tissues, and, in the following paragraphs, a few data on their occurrence in cells will be reported. (For literature see Warburg¹⁰⁰.)

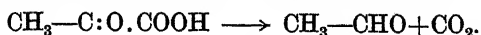
1. *Flavoproteins*. Flavin is present in a number of enzymes: *d*-amino-acid oxidase, xanthine oxidase (Scharinger's enzyme), aldehyde oxidase of mammalian liver, fumaric oxidase of yeast, Warburg's 'yellow' enzymes and diaphorase. The flavins occurring in the tissues belong to a group of pigments which are called lyochromes; they are isoalloxazine derivatives; riboflavin is known to be part of the vitamin B complex (B_2). It contains a ribose group attached to the isoalloxazine ring. As a prosthetic group of enzymes, lactoflavin is esterified with phosphate. Riboflavin (also known as lactoflavin) shows a characteristic greenish fluorescence. Ellinger and Koschra^{15,16,17} observed this fluorescence in living mammalian tissue (kidney and liver) under the fluorescence microscope. Keilin and Smith have seen in the 'empty' spectrum of an absorption spectroscopy two bands at $455\text{ m}\mu$ and $495\text{ m}\mu$ which might be due to the presence of flavin in the human retina.

Many of the flavoproteins also contain an adenine group in addition to the isoalloxazine group.

2. *Pyridinoproteins*. The enzymes of this group contain nicotinic amide (Warburg) as well as adenine. The coenzyme of alcoholic fermentation (Harden and Young^{30a}) is a prosthetic group of this kind. These substances are colourless, but they have characteristic absorption bands in the ultraviolet range. The pyridino compounds have an absorption band at $260\text{ m}\mu$. The dihydropyridine (dihydro-coenzyme) has a new band at $345\text{ m}\mu$. According to Warburg, the dihydropyridines show a marked whitish fluorescence in ultraviolet light. The pyridines do not show this fluorescence. In sections of living liver tissue small granules can be seen in ultraviolet light which show a brilliant white fluorescence. This fluorescence, which fades very quickly, may well be due to the dihydropyridines present. A great number of specific dehydrogenases contain coenzymes of this group. The pyridino group is reduced to dihydropyridine during the activity of these enzymes. One of the flavoproteins, diaphorase, acts by reoxidizing the dihydro-*l*-pyridine formed. Nicotinic acid is the pellagra-preventing factor of the vitamin B complex.

3. *Thiaminoproteins*. Ellinger and Koschra^{15,16,17} saw under the fluorescence microscope that in alkaline reaction the green fluorescence of liver tissue due to flavin disappeared and was replaced by a bluish fluorescence. We now know that this fluorescence is due to the presence of thiamine in the tissue. This substance is known to be identical with vitamin B_1 , now known as aneurin or thiamine. In combination with phosphoric acid it is a component of those enzymic systems which metabolize pyruvic acid. The metabolism of pyruvic acid in the animal

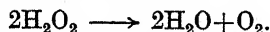
has chiefly been studied by Peters and his collaborators in Oxford. The mechanism of the reaction seems to be more complex than in yeast, where diphosphothiamine acts as co-carboxylase in the reaction⁷⁰:



c. Other Enzymes containing Heavy Metals.

1. *Iron.* The cytochrome system is not the only enzyme which contains haemin-iron. Two other systems may be briefly mentioned:

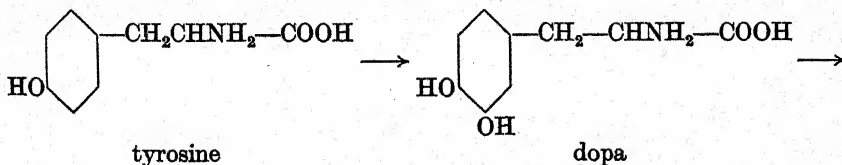
(α) *Catalase*: it catalyses the reaction:

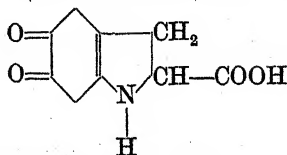


This reaction, which has been known for a long time, may be called the 'catalase' action of the enzyme; the importance of the enzyme for cellular metabolism may be due to its 'peroxidase' reaction: the oxidation of alcohol to acetaldehyde in the presence of 'nascent' peroxide. The enzyme is almost ubiquitous; only a few anaerobic bacteria are free of catalase.

(β) *Peroxidase*: it catalyses the oxidation of a number of oxidizable substrates in the presence of peroxide. It is chiefly found in plants; horse-radish usually serves as a source of the enzyme. Peroxidase has a typical absorption spectrum with bands at 645, 583, 548, and 498 m μ . Keilin and Mann⁴⁹ have seen the band at 645 m μ when they examined a slice of horse-radish root of 1 cm. thickness with a micro-spectroscope. In mammals the enzyme is present in the mammary gland and in milk. The peroxidase reaction of the granula of leucocytes consists in the formation of a coloured reaction product from a suitable substrate (e.g. benzidine) in the presence of peroxide. In spite of much work on the subject it is not known whether the peroxidase reaction of leucocytes is due to a true peroxidase or not, as the reaction is relatively stable to heating. The reaction is not given by lymphocytes.

2. *Copper* is present in a number of enzymes which oxidize phenols (monophenoloxidase, polyphenoloxidase, laccase) and in the ascorbic acid oxidase of plants. Monophenoloxidase (or tyrosinase) differs from the polyphenoloxidase in that it attacks monophenols, e.g. tyrosine; it occurs in potatoes, in certain mushrooms (*Russula*), in the meal-worm (*Tenebrio*). The reaction catalysed by the enzyme has been cleared up by the work of Raper; tyrosine is oxidized to 3, 4-dihydroxy-phenylalanine (usually briefly called dopa), and this substance is further oxidized to a red precursor of melanin:





red substance

2a. *Dopa oxidase*. In 1917 the Swiss dermatologist Bloch⁵ described a reaction of the mammalian skin. He treated unfixed frozen sections (in agar) with dilute solutions of dopa. There appeared after some time a dark pigmentation in many cells of the basal layer of the epidermis, and especially in the so-called dendritic cells, cells which usually show pigmentation. This reaction was not found in albinotic skin; it was intensified in skin previously irradiated. Bloch believed that the reaction was strictly specific and characteristic of the cells which formed pigment ('melanoblasts'), and he distinguished them from the cells of the dermis which only stored pigment and which did not give the dopa reaction ('melanophores'). Later on it was found that some cells of the dermis also gave a positive dopa reaction (e.g. the deep pigment cells of the rat's skin; the pigment cells of the so-called 'mongol spot' in man).

The dopa reaction in the embryonic eye has been studied by Miescher⁷⁵. In the chick embryo a positive reaction begins to appear at the end of the third day in the pigment epithelium of the retina, just about at the time when the pigment formation begins. The reaction becomes more intense in later stages, reaches a maximum, and decreases until it is negative again on the 18th-20th day. In the pigment cells of the choroid, the ciliary body, iris, and pecten the reaction also is first seen when pigmentation occurs and becomes negative after the pigment has been fully formed. Similar changes take place in the mammalian eye.

The theory advanced by Bloch and his collaborators was that a precursor of melanin circulated in the blood and that from it pigment was formed by the specific enzyme present in the melanoblasts. Bloch believed that dopa was this precursor, and the dopa oxidase the specific enzyme. The following points remain to be cleared up:

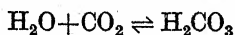
(α) *Specificity of the enzyme*. Bloch claimed that *l*-dopa was the only substrate of the enzyme. This is to-day no longer maintained: a number of other phenols (hydroxytyramine (Mulzer and Schmal-fuss⁷⁷), *p*-hydroxyphenylpyruvic acid and 3,4-dihydroxypyruvic acid (Moncorps⁷⁶)) give a similar reaction. In this connexion it must be mentioned that phenoloxidases have so far not been shown to be present in mammals. Moreover, it has already been mentioned that the cytochrome system also oxidizes polyphenols.

(β) The conception of a precursor present in blood does not explain observations of supravital formation of pigment in isolated skin *in vitro* (Meirowsky⁷⁴).

(γ) The reaction is also given by some tissue elements not connected with pigment formation, e.g. muscle, sweat gland, leucocyte granules, ganglion cells.

There seems to be little doubt that the dopa reaction reveals sites of pigment formation in the body; but how far it can really be considered as a criterion for pigment formation is not known. From the point of view of the biochemist, dopa may very well be a precursor of melanin, but this cannot be considered as proved.

3. *Zinc. Carbonic Anhydrase.* This enzyme was first discovered by Meldrum and Roughton; it catalyses the reaction



in both directions. The enzyme has been shown by Keilin and Mann^{50, 51} to be a zinc-protein compound. The importance of the enzyme for CO_2 transport in mammalian blood has been fully discussed by Roughton⁵²; the enzyme is found in the red corpuscles only; in fact, in some species the plasma contains an inhibitor for the enzyme (Booth⁶).

In the mammal, carbonic anhydrase is also found in the gastric mucosa (Davenport and Fisher¹²). Keilin and Mann have obtained a preparation of the enzyme from pig's stomach which was probably about 30 per cent. pure; they showed that not more than 0.75 per cent. of its activity could have been present in the red blood corpuscles of the mucosa. Davenport^{9, 10, 11} found that the fundic region of the mucosa had a much higher activity than the pyloric region. As the oxyntic cells of the stomach occur in the fundus only, the correlation between enzymic activity and distribution of oxyntic cells was studied. Davenport adapted the method of Linderström-Lang (see below) for the determination of carbonic anhydrase. The gastric mucosa of rats and cats were used. From his findings Davenport concludes that the enzyme is localized in the oxyntic cells and that they contain 5 to 6 times as much enzyme as the red blood corpuscles. The peptic cells of the gastric glands were found not to contain the enzyme; the cells of the gastric surface epithelium have about one-tenth of the activity of the oxyntic cells. Davenport has since confirmed his observations for the gastric mucosa of the dog.

The oxyntic cells are believed to be responsible for the secretion of hydrochloric acid. Attempts have been made recently to find out whether carbonic anhydrase is connected with the gastric secretion of acid. According to Davenport, carbonic anhydrase is inhibited by sodium thiocyanate (NaCNS). The effect of thiocyanate on gastric acid secretion was studied in dogs, and it was found that thiocyanate inhibits secretion

of HCl. This result was confirmed by Feldberg, Keilin, and Mann¹⁹, but it was shown that a much stronger inhibitor of carbonic anhydrase, sulphanilamide, did not inhibit the secretion of acid. Although, according to the authors, these experiments do not exclude the possibility that carbonic anhydrase may have an indirect connexion with the secretion of hydrochloric acid, they do not support the view that the enzyme directly catalyses the secretion of HCl. The question why there is so much enzyme to be found in the oxyntic cells remains to be answered.

Recent observations of the occurrence of carbonic anhydrase in teleost fishes are of interest. In 1932 Keys and Willmer⁵² described in the gills of the eel (*Anguilla vulgaris*) and other teleosts eosinophil cells similar in appearance to the oxyntic cells of the gastric mucosa. These cells are secretory cells; they are situated in the proximal parts of the gill leaflets, especially on the side nearest to the gill rays (see Plate I, Fig. 1). They are therefore in close contact with the venous blood entering the gills. Keys and Willmer ascribe to these cells, especially in marine species, the function of excreting chloride from the blood into the seawater. Leiner^{57, 58} describes these cells again and finds them not only in the gills but many more of them in the pseudobranchs, a preponderantly glandular structure situated on the roof of the palate. The organ contains a vascular network which gives its blood to the major ophthalmic artery. Leiner finds a very high carbonic anhydrase activity in the pseudobranchs; the gills also contain the enzyme. He believes that the enzyme occurs in the eosinophil cells and that they secrete the enzyme into the blood. However that may be, it is interesting that these two types of cells of similar appearance, the oxyntic cells of the gastric mucosa and the eosinophil cells in teleosts, should contain carbonic anhydrase in high concentration. Carbonic anhydrase is also found in the teleostian eye (choroid, choroid body, retina).

In the invertebrates, carbonic anhydrase occurs also in the gills (lobster, squid). *Limulus* contains the enzyme in the white cells. (Robertson and Ferguson⁸⁷; Ferguson, Lewis and Smith²⁴; van Goor³⁰)*

iii. HYDROLYSING ENZYMES

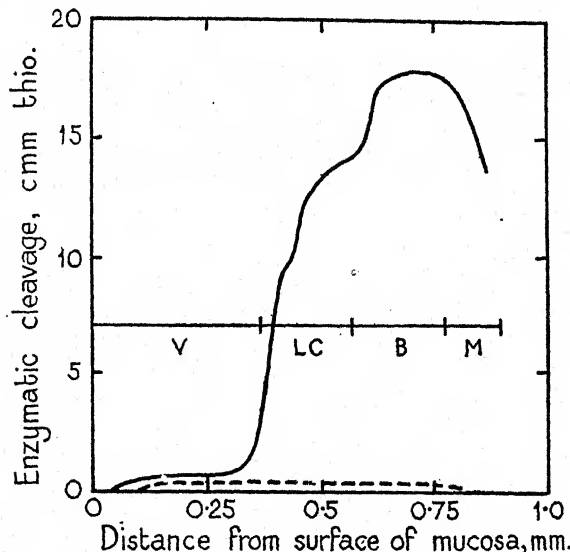
Linderström-Lang⁵⁹⁻⁶⁹ and his collaborators in Copenhagen have worked out micro-methods for the determination of a number of enzymes in microtome sections (c. 25μ thick). The principles of these methods were described by Linderström-Lang^{60a} and Holter⁶³. The fresh tissue is frozen and a cylinder of a diameter of 2.36 mm. is stamped out and cut on a freezing microtome. Alternate sections can be used for the enzyme analysis and for the histological control.

* Keilin and Mann (*Nature*, 1941, 148, 493) have recently described a method by which the role of the enzyme in the transport of carbon dioxide in the red corpuscles can be made visible.

a. Carbohydrate-splitting Enzymes.

1. *Amylase.* Linderström-Lang and Engel⁶² analysed the distribution of amylase in the cells of the barley grain. The cells at the border of aleuron and starch cells showed the highest content of this enzyme, about 20 per cent. of the total amylase content of the grain.

Van Genderen and Engel²⁷ in Utrecht studied the distribution of this enzyme in the mucous membrane of the gut. Horizontal sections



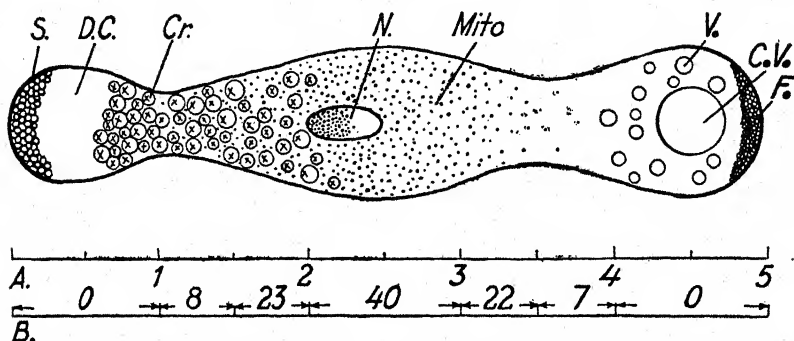
TEXT-FIG. 3. Amylase activity in the duodenal mucosa of the rabbit. Ordinates: c.mm. $N/20 Na_2S_2O_3$. Abscissae: V = level of villi; LC = level of crypts of Lieberkühn; B = level of Brunner's glands; M = level of muscle coat. After van Genderen and Engel²⁷. — amylase activity; ---- blanks.

through the wall of the gut, i.e. parallel to the free surface, contained varying amounts of amylase, dependent on the level of the section. The first sections through the block of tissue contained the villi only; the following sections went through the region of the crypts of Lieberkühn; still deeper sections cut through the muscularis mucosae, and the last sections through the submucosa, which in the case of the duodenum contains Brunner's glands. It was found that in the rabbit the sections through Brunner's glands in the submucosa contained the largest amount of amylase. Brunner's glands of the rabbit contain serous and mucous cells, whereas those of the rat contain mucous cells only; in this animal no amylase has been found in the level of the glands.

The enzymic activity was measured by incubating a 25μ section of a diameter of 2.36 mm. for 40 minutes at $32^\circ C$. with a standard amount of starch. At the end of the experiment the amount of starch left over was determined iodometrically. The curve in Text-fig. 3 shows the

amount of $N/20 Na_2S_2O_3$ used for the free iodine. Thus the curve expresses directly the enzyme activity in the sections.

In the ileum of the rat amylase was found only in the region of the villi, smaller amounts in fasting animals, more after feeding. It is very likely that the enzyme is only adsorbed to the surface of the epithelial cells covering the villi, but not produced by them.



TEXT-FIG. 4. Diagram of centrifuged amoeba. The first scale shows the division in transverse zones; the second scale the percentage of mitochondria found in each zone. For abbreviations see text. After Holter and Doyle³⁵.

The distribution of amylase in the cytoplasm was investigated by Holter and Doyle³⁵ in the case of *Amoeba proteus*. When an amoeba is centrifuged it is stretched and the cell contents arrange themselves in layers.

Text-fig. 4 shows the stratification: fat granules (F.) and vacuoles (V. and C.V.) accumulate near the centripetal pole, dense cytoplasm (D.C.), crystalline (Cr.), and some other cytoplasmic inclusions (S.) near the centrifugal pole. The nucleus retains a central position and a broad middle zone is filled with mitochondria (Mito.). It is this region which contains the amylase as determined by sections at right angles to the long axis of the cell. The enzyme content in each section is proportional to the amount of mitochondria, e.g. zones 1 to 3 contain 71 per cent. of the total amylase and zones 1 to 4 all the amylase of the cell. It is therefore assumed that the mitochondria are the carriers of this enzyme. This is particularly interesting because we know from other investigations which will be mentioned later that, for example, peptidase (and also catalase) are diffusely distributed in the cytoplasm and do not appear to be attached to specific structures.

2. *Maltase*. Van Genderen and Engel²⁷ also investigated the distribution of maltase in the ileum of the rat. In the fasting animal they found most of the enzyme in the sections through the villi and also through the upper parts of the crypts of Lieberkühn, but in the ileum of a rat killed 50 minutes after feeding the crypts of Lieberkühn do not contain this

enzyme. If enzymes were formed in the intestinal mucosa one would expect to find at least the same amount of enzyme after feeding. These results therefore suggest another explanation: that the enzyme is of pancreatic origin and is only adsorbed on the epithelial cells of the villi and upper part of the crypts. On the other hand, it is interesting to note that in fishes which have no crypts of Lieberkühn, maltase has not been found in the intestinal mucosa (Vonk⁹⁵).

b. Lipolytic enzymes.

1. *Lipase.* The distribution of lipase in the mucosa of the pig's stomach and duodenum has been investigated by Glick.²⁸ He also used Linderstrøm-Lang's technique. Lipase was found to occur in the mucous membrane of the cardia, fundus, pylorus and duodenum, in approximately equal concentrations. The prismatic cells lining the surface and the gastric pits in the three parts of the stomach as well as the glands in the cardiac and pyloric region contain the enzyme. The glands of the fundus show a slightly smaller activity. All parts of the duodenal mucosa contain a fairly high concentration of lipase; the greatest activity is found in the villi of the duodenum.

The action of the enzyme was measured by titrating the acid formed from 1 per cent. methyl butyrate during 5 hours at 40° C. by a 25 μ section of 2.36 mm. diameter. Linderstrøm-Lang and Holter⁶⁸ define the activity of this esterase as follows: one esterase unit is the amount of enzyme which splits 0.5×10^{-4} milliequivalent ester linkages in 14 c.mm. 0.5 per cent. methyl butyrate (pH = 8.7) in 5 hours at 40° C. Sections through the surface cells and gastric pits contain about 2 enzyme units; slightly lower values were obtained for the cardiac and pyloric glands. The lipase activity of the glands of the fundus is about half as high. Between 3 and 4 units were found in sections through the duodenal villi. The lipase curves in Text-fig. 6 (p. 210) show the relative amounts of this enzyme in the layers of the mucosa of stomach and duodenum.

The results obtained by Jeker⁴³ are of interest in this connexion. He used histochemical methods in his investigation of fat resorption in the small intestine of rats, especially two histological methods for frozen sections: (1) Sudan III which stains neutral fats well; (2) Fischler's²⁵ method which shows the presence of fatty acids, especially oleic acid, but also palmitic and stearic acids, and which is based on the fact that these acids form soaps when treated with cupric acetate. The copper soaps form black lakes with haematoxylin.

The epithelial cells covering the villi contain a considerable amount of black droplets and granules when treated by Fischler's method 30 minutes after feeding 3 c.c. olive oil. Very little fat can be shown with Sudan III at this stage. But a few hours later the reverse is observed:

only traces of fatty acid are found in the cytoplasm of the epithelial cells; they now contain a large number of fat droplets which stain intensely with Sudan III. In other words, the action of the lipase in the epithelial cells can be observed: the resorbed fatty acids are resynthesized with glycerol to neutral fats in the cytoplasm of the cell. Figures 2, 3, 4, and 5, Plate I, show the fatty acid in the cells. Figures 2 and 3 show a villus 30 minutes and 6 hours after feeding with oil, and Figures 4 and 5 show a single cell at the same phases respectively. The large 'vacuoles' in the cytoplasm of the cells of Figures 3 and 5 are droplets of neutral fat which stain well with Sudan III.

At least one more enzyme (a phosphatase) seems to take part in the resynthesis of neutral fats in the epithelium of the villi. The evidence for this will be described later.

It may also be mentioned here that, by centrifuging, Behrens³ was able to separate cytoplasm and nuclei of liver cells. He found the activity of lipase 20 times higher in the cytoplasm than in the nuclei. The small amount of lipase in the nuclei is probably due to an incomplete separation of cytoplasm and nuclei. The details of the method will be given later.

c. *Proteolytic enzymes.*

1. *Pepsin.* The distribution of pepsin in the mucosa of the pig's stomach was investigated by Holter and Linderstrøm-Lang⁵⁸ by using horizontal sections through the mucous membrane. They used edestin (2 per cent.) as a substrate and measured the amino groups set free by the enzyme after 2 hours at 40° C. (pH 2.1) by titrating with N/20 HCl in acetone. This method was described by Linderstrøm-Lang⁵⁹. 1 c. mm. N/20 HCl corresponds to 0.7 μ g. amino nitrogen. In the following paragraph the enzyme activity will be expressed in enzyme units, defined by Linderstrøm-Lang and Holter⁶⁸ as the amount of pepsin which splits 0.63×10^{-4} milliequivalent peptide linkages of 2 per cent. edestin (pH = 2.1) in 14 c. mm. during 2 hours at 40° C. 1 pepsin unit corresponds to 7×10^{-4} mg. Parke-Davis pepsin 1:10,000 or 1.4×10^{-4} mg. crystalline pepsin.

A very high activity was found in sections through the fundus, especially the sections which pass through the deeper part of the tubular glands, where most of the 'peptic' cells are found. The enzymic activity corresponds here to 32 or even 40 pepsin units.

The pyloric mucosa shows a much smaller activity; pepsin was found especially in the deeper sections through the glands and amounted to just over 4 pepsin units (Linderstrøm-Lang and Holter⁶⁸). The 'pepsin curves' in Text-fig. 6 give the distribution of the relative enzyme activities in the three parts of the stomach. It will be noted that a proteinase (pepsin?) which splits edestin at pH 2.1 was also found in

Brunner's glands in the duodenum; it occurs there in concentrations similar to those in the cardiac glands.

Pepsin is an enzyme which has been prepared in a crystalline state. It can therefore be calculated that a single peptic cell in a gland of the fundus contains about 8 per cent. of its weight (c. 3×10^{-6} mg.) of pepsin. If all pyloric gland cells were engaged in pepsin production the pepsin concentration in each single glandular cell would be about one-tenth of the fundus value.

Pepsin units in a single cell in stomach and duodenum

<i>Cardia</i>	<i>Fundus</i>	<i>Pylorus</i>	<i>Duodenum</i>
Very low in all layers	Surface epithelium and gastric pits 0.2	Surface epithelium and gastric pits 0.07-0.11	Epithelium of villi and crypts 0
	Cell of the neck of the glands 0.2-1.2	Cell of the neck of the glands 0.09-0.16	
	Peptic cell 1.7-2.1	Pyloric gland cell 0.14-0.23	Brunner's gland cell 0.04
	Oxyntic cell 0		

Production of hydrochloric acid. Heidenhain³¹ suggested that the oxyntic (parietal) cells in the glands of the fundus produce hydrochloric acid and that the smaller, 'chief' or peptic cells, produce pepsin. Langley *et al.*⁵⁴⁻⁵⁶ brought further evidence in favour of this suggestion. They investigated the frog's stomach where, to a certain degree, glands producing pepsin and hydrochloric acid are separated from each other.

The final proof that HCl is produced by the oxyntic cells was given by Linderstrøm-Lang and Holter^{66,67}. They made a detailed quantitative study of the acid content of horizontal sections through the mucous membrane of the pig's stomach, which is rather similar to that of the human stomach. Most of the oxyntic cells are found in the upper part of the glands of the fundus to a depth of c. 1 mm., and the peptic cells are mainly found in the lower part of these glands. The maximum of acid corresponds to the level of highest frequency of oxyntic cells. The curves for acid and pepsin content are quite different from each other and correspond very well to the distribution of the two types of cells (Linderstrøm-Lang, Holter, and Sæborg Ohlsen⁶⁹).

The acid in 50 μ sections of 2.36 mm. diameter is neutralized by 2.5 cm. M/100 borate. This amount of acid if distributed evenly through an oxyntic cell would amount to a concentration of 0.15 N;

it is most probable that such an enormous concentration can be present only as bound acid.

Little is known about the mechanism which leads to the liberation of the acid, but the following two points seem to be of particular interest in this connexion: (1) the high content of carbonic anhydrase in the glands of the fundus, most probably in the oxyntic cells (see p. 199) and (2) the investigation by Palmer (see Linderstrøm-Lang, Holter, and Sjøeborg Ohlsen⁶⁹, p. 33), who determined the chloride content of the different levels of the fundus mucosa. The following results, expressed in c.mm. N/100 AgNO₃ per 245 c.mm. tissue, were obtained: surface epithelium 1 c.mm., level of maximum of oxyntic cells (c. 0.5 mm. deep) 2.5 c.mm., bottom of the glands, corresponding to the maximum of peptic cells (1.5 mm. deep) 1 c.mm.

Digestion of Keratin. In most animals keratin is not attacked by proteolytic enzymes. The larvae of the clothes-moth (*Tineola biselliella humm*) feed chiefly on keratin. The digestion of keratin in these animals was studied by Linderstrøm-Lang and Duspiva⁶¹ and Duspiva¹⁴. The larvae of the wax-moth (*Galleria mellonella*) which does not digest keratin served for comparison. The surprising observation was made that the proteinase of the intestine of the larvae of *Tineola* does not digest keratin *in vitro* at the physiological pH (9.6–10.2), though it digests casein actively. The question arose as to how the epithelial cells of the intestine were enabled to digest keratin. This was cleared up in the following experiments. The larvae were fed with wool which had been soaked in various oxidation-reduction indicators. Indigo-disulphonate stained the epithelium and the contents of the fore- and hind-gut blue, but in the mid-gut it was partly reduced to a pale green ($E_0 = -0.22$ volts). The fully reduced indicator is yellow. When cotton fibres soaked with indicators were fed the results were even clearer, as cotton is not attacked by the enzyme. In this case blue gallophenine was reduced by the cells of the mid-gut, corresponding to $E_0 = -0.29$ volts. Brilliant alizarin blue ($E_0 = -0.34$ volts), on the other hand, showed no colour change throughout the intestine. From these observations it can be concluded that the epithelial cells of the mid-gut of the larva of the clothes-moth can maintain a reduction potential as low as -0.3 volts.

It is not quite clear whether this potential is due to the presence of SH-groups only or whether other reducing agents (hydrogen carriers) are involved in this process. The important effect of this reduction potential is that the S-S linkages characteristic of the keratin molecule are reduced to —SH groups. The S-S groups form a cross-link between the single peptide chains of the protein. They are responsible for the insolubility of keratin. The reduction therefore enables the proteinase to attack the keratin. Titration of the free amino and carboxyl groups shows that the proteinase of the intestine of the *Tineola* larva splits

peptide linkages. It differs from other proteinases, e.g. that of the pancreas or that found in the wax-moth, in that it is not inhibited by free —SH groups.

2. *Peptidases*. Peptidases in cells may have different functions and consequently their localization in the cells varies considerably:

Endoenzymes, i.e. peptidases which occur in actively growing cells, where they play their role in building up proteins. In this case they are always diffusely distributed in the cytoplasm, e.g. in unfertilized or fertilized ova or growing roots of plants.

Exoenzymes, i.e. those peptidases which are found attached to specific cell granules, e.g. in the pancreas and intestine where their role may be either to be excreted or to synthesize specific proteins, e.g. other enzymes.

(α) *Peptidases in growing cells*. The dipeptidases of the ova of the marine invertebrates are endoenzymes. They will not act on dipeptides in the surrounding medium unless the cell is killed and the enzyme allowed to diffuse into the medium where it comes into contact with the substrate. The following results refer to the alanyl-glycine dipeptidase, unless otherwise stated. The unfertilized egg of *Dendraster excentricus* weighing 0.5 μ g. is able to split 1 μ g. alanyl-glycine in 1 hour at 40° C. (Linderström-Lang and Holter⁶⁵). If the egg is cut in two halves so that one contains the nucleus, both parts show an approximately equal dipeptidase activity. From this experiment it can be concluded that at least most of the dipeptidase, if not all, is in the cytoplasm. That the cell nucleus is not essential for the functioning of dipeptidase was also shown by Holter and Kopac³⁹, who denucleated amoebae and found that this had no effect on the dipeptidase activity.

The localization of dipeptidase in the cytoplasm was investigated by Philipson⁸⁴. He centrifuged unfertilized eggs of the sea-urchin *Psammechinus miliaris*. The eggs were suspended at the boundary between sea-water and sugar solution of a higher specific weight. The eggs separated into a small heavier and a large lighter part. Though the volumes were in the ratio 1:4 both parts had an equal dipeptidase activity. This shows that the enzyme molecules are associated mainly with the denser part of the cytoplasm. But it is not possible to connect them with any specific cytoplasmic structure, e.g. mitochondria. Similar results were obtained by Holter^{33,34} for the eggs of *Arbacia*, *Echinarachnius*, and *Chaetopterus*.

That fertilization does not lead to an increase of dipeptidase was shown for *Urechis caupo* by Linderström-Lang and Holter⁶⁵. The unfertilized ovum has a dipeptidase activity which is of about half the value given for *Dendraster*. Fifteen minutes after fertilization there is a slight drop in dipeptidase activity. The cause of this is obscure, but it indicates that the head of the sperm does not contain dipeptidase.

Three hours after fertilization, i.e. in the 16-cell stage, the original value has been reached, but later, 20 to 30 hours after fertilization, with decreasing growth rate, the total enzyme activity drops again to half the original value.

Doyle¹³ confirmed that fertilization does not influence the dipeptidase content in the case of the eggs of *Psammechinus miliaris*, which have a similar activity to those of *Urechis*.

The question arose whether enzyme activity and cell determination could be correlated (Needham⁸¹). Holter, Lanz, and Linderstrøm-Lang³⁶ investigated the 8-cell stage of *Psammechinus miliaris* for its dipeptidase content. At this stage the 4 upper cells are already determined to ectoderm cells and the 4 lower ones will give rise to the endodermal and mesodermal structures. But in this case no significant differences in dipeptidase activity were found between the blastomeres. Further work on these lines seems to be very promising.

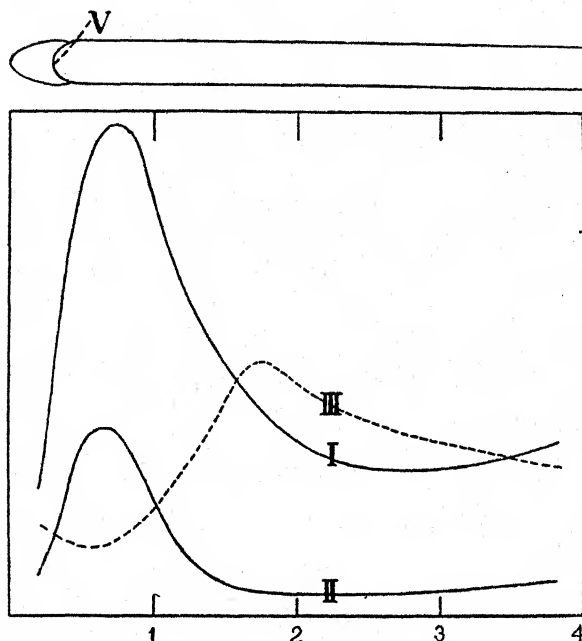
About the occurrence of other dipeptidases not much is known. Holter, Lehmann, and Linderstrøm-Lang³⁷ investigated the leucyl-glycine peptidase in the eggs of *Tubifex rivulorum*. This enzyme is activated by Mg ions. During 20 hours at 40° C. the enzyme content of one egg is able to set free amino groups corresponding to 1 c.mm. N/20 HCl. After the addition of Mg ions this value rises to 3 to 4 c.mm. No activation by Mg was observed with the alanyl-glycine peptidase; its activity corresponded to 1 to 4 c.mm. N/20 HCl with and without the addition of Mg.

In the radicle of the barley germ (Text-fig. 5) these two peptidases occur in different concentrations at different distances from the tip of the root (Linderstrøm-Lang and Holter⁶⁴). The maximum of both enzymes is found at a distance of 0.8 mm. from the tip of the root, i.e. not at the point of maximum of cell division (V) but in the region of very active longitudinal cell growth. Mitotic divisions are found mainly in the first half mm. The ratio alanyl-glycine peptidase to leucyl-glycine peptidase changes in favour of the former with the distance from the tip of the root (see curve III, Text-fig. 5). This suggests that the cytoplasm of the older (longer) cells contains more alanyl-glycine groups than that of the less differentiated, younger cells.

The alanyl-glycine peptidase in a 200 μ section through the root containing c. 0.014 c.mm. tissue is able to split about 10^{-7} mol alanyl-glycine during 5 hours at 40° C. On the other hand, in the leaf germs both enzymes are evenly distributed, and here zones of cell division and cell growth cannot be distinguished.

(β) *Peptidases of stomach, intestine, and pancreas.* (i) *Stomach.* The alanyl-glycine peptidase is not found in the gastric juice but in the mucous membrane of the three parts of the stomach. It is found in the duodenal juice and in the duodenal mucosa.

Linderstrøm-Lang and Holter^{66, 67} investigated the distribution of the alanyl-glycine peptidase in the mucosa of stomach and duodenum of the pig. Details of dipeptidase distribution in cardia, fundus, and pylorus can be seen in Text-fig. 6. In the mucosa of the fundus the distribution of this enzyme corresponds to the distribution of the peptic cells.

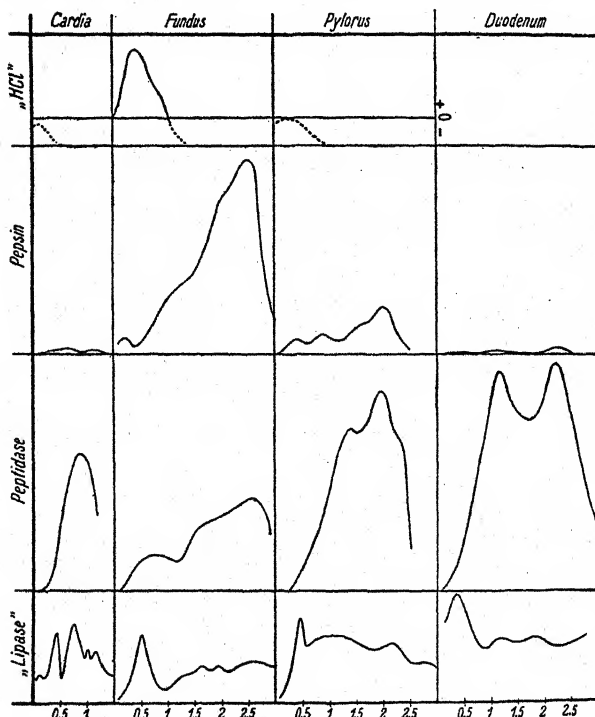


TEXT-FIG. 5. Distribution of I, alanyl-glycine dipeptidase, and II, leucylglycine dipeptidase, in the radicle of the barley germ. Ordinates enzyme activity; abscissae: distance from tip of the root in mm. Curve III shows the ratio of both enzyme activities. After Linderstrøm-Lang⁶⁶.

As the peptidase content in the 25μ sections (diameter 2.36 mm.) was considerable, measurements of the enzymic activity were made for a period of 20 minutes at 30° C. only. The amino groups set free were titrated with $\text{N}/20 \text{ HCl.}$

The activity of the enzyme is expressed in units defined as follows: one unit is the amount of enzyme which splits 0.5×10^{-4} milliequivalent peptide linkages in 14 c.mm. 0.1 M alanyl-glycine solution at $\text{pH } 7.4$ in 20 minutes at 30° C. In one 25μ section the following values were found: cardia, maximum of nearly 4 units in the glands; fundus, maximum of about 3 units in the region of the peptic cells of the glands; pylorus, maximum of over 5 units in the region of the glands; in the duodenum maxima of nearly 6 units, both in Brunner's glands and in the crypts. For further details of dipeptidase distribution see Text-fig. 6.

The deeper levels of the glands of the fundus, where most of the peptic cells are found, shows a 10–20 times higher activity of peptidase than of pepsin. Linderstrøm-Lang, Holter, and Søbørg Ohlsen⁶⁹ calculated the dipeptidase activity of a single peptic cell of the glands of the fundus; it is able to split 4.5×10^{-7} mg. alanyl-glycine in 20



TEXT-FIG. 6. Diagram of relative enzyme activities (ordinates) of the mucosa of the pig's stomach. Abscissae: distance in mm. from the surface. For details see text. After Linderstrøm-Lang⁶⁹.

minutes at 30° C., i.e. 0.15 times its own weight. This activity is about half that of the rapidly growing ovum of *Dendroaster* which splits 0.3 times its own weight in similar conditions.

Similarly the dipeptidase activity of a single pyloric gland cell under the same conditions can be calculated. The cell hydrolyses 0.68 times its own weight of alanyl-glycine, i.e. the dipeptidase activity is 4.5 times greater than in the peptic cells of the fundus glands. On the other hand, it will be remembered that the cells of the pyloric glands contain 10 times less pepsin than the peptic cells of the glands of the fundus. There does not seem to be any clear correlation between these two enzymes.

A dipeptidase for glycyl-l-proline was found in the pig's stomach. The enzyme is present in all layers of the fundic and pyloric mucosa.

Its activity varies between 2 and 3 enzyme units. The proline peptidase unit as used by Linderstrøm-Lang and Holter⁶⁸ is that amount of enzyme which splits 0.5×10^{-4} milliequivalent peptide linkages in 14 c.mm. 0.1 M glycyl-L-proline at pH 7.4 in 5 hours at 40° C.

No carboxypolypeptidase is found in the stomach, but a fairly active aminopolypeptidase which occurs in fundus and pylorus in a distribution similar to that of the alanyl-glycine dipeptidase. The activity of the aminopolypeptidase corresponds to 4 to 5 enzyme units defined as that amount of enzyme which splits 0.5×10^{-4} milliequivalent peptide linkages in 14 c.mm. 0.1 M alanyl-glycyl-glycine at pH 7.3 in 20 minutes at 30° C. (Linderstrøm-Lang and Holter⁶⁸).

A scheme of reactions for these enzymes is given on p. 215.

(ii) *Intestine*. For the mucous membrane of the pig's duodenum the following results were obtained. There are two maxima in the distribution of alanyl-glycine dipeptidase activity: one coincides with the crypts of Lieberkühn, the other with Brunner's glands. The alanyl-glycine dipeptidase activity of 25 μ sections at the level of Brunner's glands and at the level of the crypts each amounted to 4-6 units.

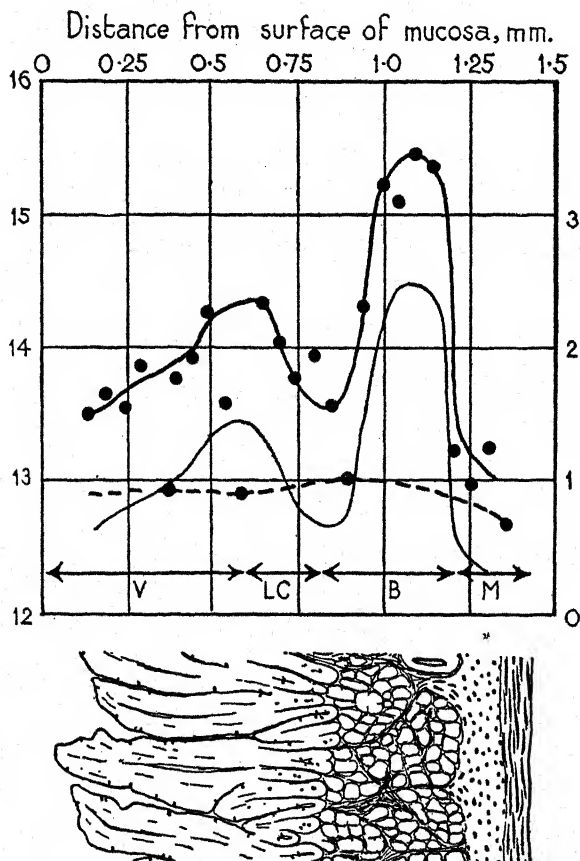
The peptidase content of a single cell of Brunner's glands is of the same order as that of a cell of the pyloric and cardiac glands (c. 0.3 units).

Similar results with glycyl-glycine as substrate were obtained by van Genderen and Engel²⁷, who used the duodenum of the rat (Text-fig. 7). The same authors and van Weel⁹⁷ also studied the distribution of the dipeptidase in the ileum of the rat. Here the crypts are longer and allow a more detailed analysis, while Brunner's glands are absent. The distribution again shows two maxima: the one is found in the villi; the other in the deepest part of the crypts, where Paneth cells occur (Text-fig. 8 and Plate II, Fig. 1). The interpretation given by the author is that the production of the enzyme takes place in the Paneth cells, which extrude their granules into the lumen of the crypts, where they dissolve rapidly. The surface of the villi merely adsorbs the enzyme. In the light of these conclusions it would be interesting to analyse the small intestine of dogs and cats, where Paneth cells do not occur. The granules of the Paneth cells are regenerated c. 7 hours after an injection of pilocarpine, but an analysis of the parallelism between the number of granules in Paneth cells and their enzyme content has not yet been made. The granules themselves give a positive ninhydrin reaction (Hintzsche and Anderegg³²). Even if all prismatic cells were engaged in the production of the enzyme the dipeptidase content of a single cell would be the highest so far recorded, corresponding to c. 0.8 units.

(iii) *Pancreas*. The occurrence of two types of peptidases in the excretory cells of the pancreas was analysed by van Weel and Engel⁹⁹

and van Weel⁹⁷: these are glycyl-glycine dipeptidase and carboxy-polypeptidase.

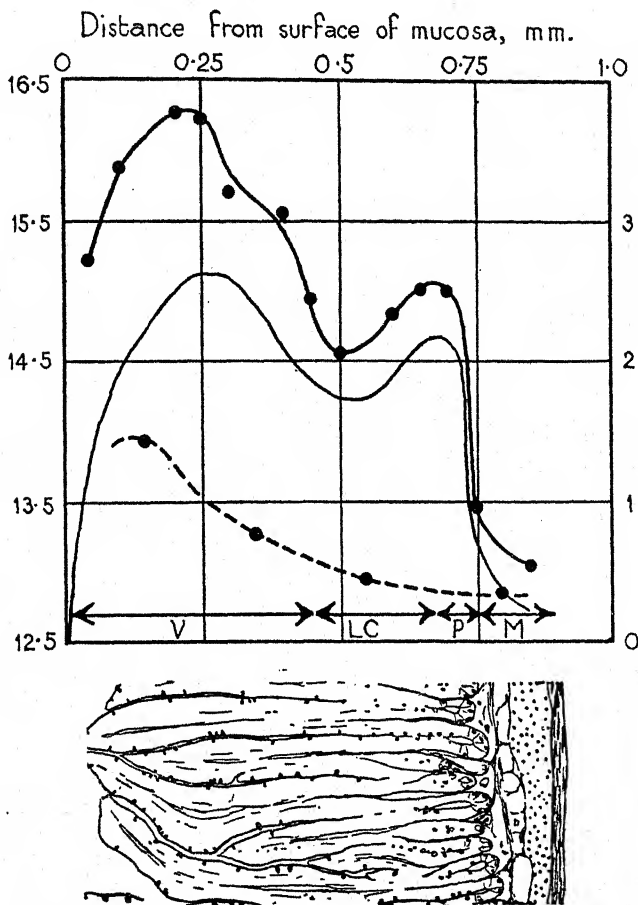
The pancreatic secretion contains no dipeptidase under physiological conditions. Only if a very strong stimulus is applied, e.g. intravenous



TEXT-FIG. 7. Glycyl-glycine dipeptidase activity in the upper duodenum of the rat. Values after 3 hours at 37° C. Curves: *a*, thick line: total acid used in c.m.m. N/5 HCl (left ordinate scale); *b*, dotted line: blanks (left ordinate scale); *c*, thin line: difference between *a* and *b* in c.m.m. N/5 HCl (right ordinate scale). The thin curve expresses the enzyme activity. The diagram shows a longitudinal section through the upper duodenum. V, Villi; LC, Crypts; B, Brunner's Glands; M, Muscularis (van Genderen and Engel⁹⁷).

injection of pilocarpine, do traces of dipeptidase appear in the secretion. But this enzyme can always be found in the cells. The amount of enzyme in a 25 μ section (diameter 2.6 mm.) of the pancreas of a fasting rat is able to split peptide linkages corresponding to 0.4 c.m.m. N/5 HCl during 2 hours at 37° C. One hour after injection of pilocarpine this

value has dropped to 0.3 c.mm. and 5 hours after the injection it reaches a maximum of 0.55 c.mm. (Text-fig. 9). Later the activity drops

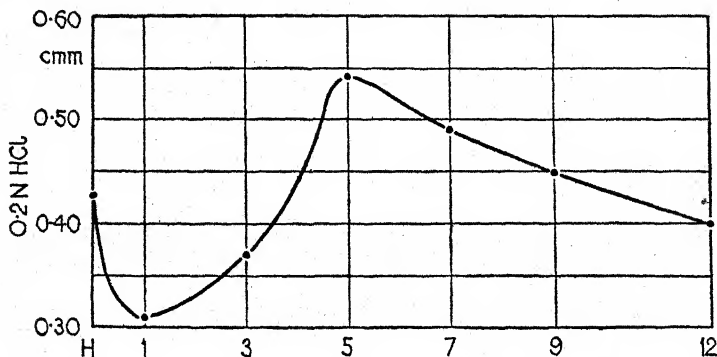


TEXT-FIG. 8. Glycyl-glycine dipeptidase activity in the ileum of the rat. Abscissae and ordinates as in Text-fig. 7. P = level of Paneth cells, other abbreviations as in Text-fig. 7. After van Genderen and Engel²⁷.

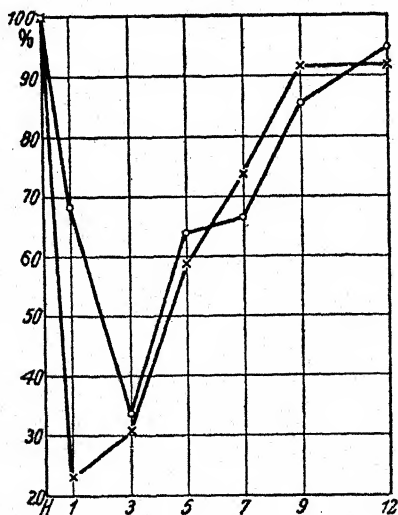
gradually until it reaches the resting value 9–12 hours after the injection (van Weel⁹⁷).

The interesting point about the regeneration of the dipeptidase is the following: the granules of the pancreatic cells are extruded into the lumen of the acini after an injection of pilocarpine; they reach a minimum in the cells about an hour later and are gradually reformed until, after about 12 hours, the original amounts are present (curve x—x, Text-fig. 10). Dipeptidase is not present in the granules which are excreted; the presence of very small amounts of dipeptidase in the

pancreatic juice after an unusually strong stimulus is due to the very rapid extrusion of the cell granules which under these conditions carry



TEXT-FIG. 9. Glycyl-glycine dipeptidase activity in the pancreas of the mouse. Ordinates: c.mm. N/5 HCl due to enzyme activity. Abscissae: hours after injection of pilocarpine. H = value for the fasting animal. (van Weel⁹⁷.)

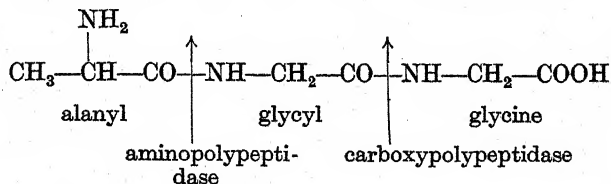


TEXT-FIG. 10. Granula content (x-x) of pancreatic cells of the mouse and carboxy-polypeptidase content (o-o) of the pancreas expressed in percentages of the values for the fasting animal (H = 100 per cent.). Abscissae: hours after injection of pilocarpine. (van Weel and Engel⁹⁸.)

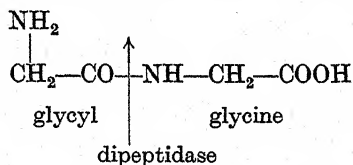
with them small particles of cytoplasm. This has been confirmed by histological observation.

Van Weel⁹⁷ suggests the hypothesis that the dipeptidase plays a role in the formation of another enzyme: the carboxypolypeptidase which is normally secreted by the pancreas cells. This enzyme has been prepared in a crystalline state by Anson¹. Its action is as follows: it splits

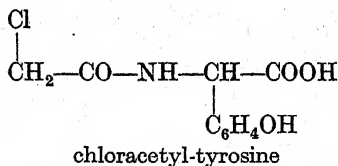
the peptide linkage next to a free COOH group provided there is no basic group in its vicinity; e.g. the tripeptide alanyl-glycyl-glycine is split at the glycyl-glycine link, not at the alanyl-glycyl link.



The latter can be split only by an aminopolypeptidase. On the other hand a dipeptide, e.g. glycyl-glycine, cannot be split by carboxypolypeptidase or by aminopolypeptidase, because both a free amino and carboxy group are close to the peptide linkage. This linkage can

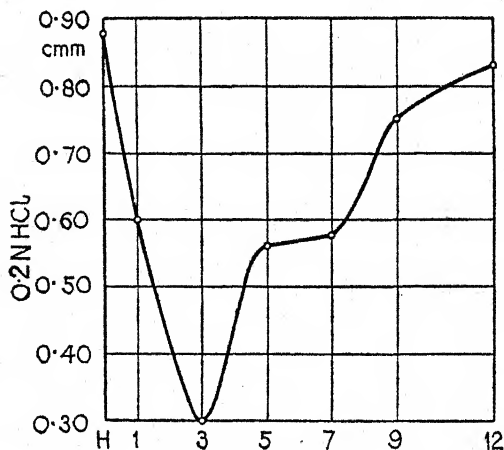


be split only by a dipeptidase. Chloracetyl-tyrosine, which contains no free amino group, can therefore be used as a substrate for carboxypolypeptidase.



Van Weel and Engel⁹⁹ were able to show that there is a definite correlation between the amount of granules in the pancreatic cells and the carboxypolypeptidase content determined in sections. The highest content of enzyme is found in a fasting animal. One hour after an injection of pilocarpine a sharp drop occurs to two-thirds of the initial activity and to one-third after 3 hours (Text-fig. 11). Later the enzyme content gradually rises again to reach its initial value after c. 12 hours. The number of granules in the cells shows a similar change: the highest content of granules is found in the cells of the resting animal; it amounts to about 35 per cent. of the weight of the cell. One hour after pilocarpine the granules represent only 10 per cent. of the cell weight, but regeneration of granules begins soon afterwards. The initial value is reached after 9-12 hours. In the curves of Text-fig. 10 the number of cell granules and the enzyme activity of the fasting pancreas are taken as 100 per cent.; it can be seen that a striking parallelism exists between the two curves. The time-lag in the fall of the enzyme content during

the 1st to 3rd hours is due to the accumulation of secreted enzyme in the ducts of the gland which have practically no muscle in their wall and empty their contents only slowly.



TEXT-FIG. 11. Carboxypolypeptidase activity in the pancreas of the mouse. Ordinates and abscissae as in Text-fig. 9 (van Weel and Engel⁹⁹).

Practically nothing is known about the synthesis of enzymes in cells. But two points may be of interest in this connexion:

- (1) By comparing Text-figs. 9 and 11 it will be seen that the rise of the dipeptidase curve, which reaches its maximum during the 5th hour, always precedes that of the carboxypeptidase curve. It is mainly on these grounds that van Weel suggested the possible role of the dipeptidase in the formation of the carboxypolypeptidase.
- (2) Van Weel⁹⁶ reports that during the regeneration of the cell granules, c. 1-2 hours after feeding, small granules are found in the cytoplasm which give a positive Schiff reaction after acid hydrolysis. The desoxypentose of the nucleic acid gives the same aldehyde test, and van Weel suggests that possibly nucleic acids or their derivatives play a role in these processes.

(d) *Urease.*

The distribution of urease in the layers of the mucosa of the dog's stomach was investigated by Linderstrøm-Lang and Søbørg Ohlsen^{88a}. The enzyme was found to be present in the prismatic cells lining the surface and the gastric pits in fundus and pylorus and also in the cells forming the necks of the glands in both parts of the stomach. The absolute values of urease activity showed considerable variation in the three stomachs analysed. The maxima were found in the fundus at a

depth of 0.2 to 0.4 mm.; this corresponds to the gastric pits or the neck of the glands, and the titration values for the liberated ammonia were between 0.5 and 12 c.mm. N/20 HCl after 90 minutes' incubation of two 25μ sections. The maxima in the pyloric region were at 0.4 mm. depth, which corresponds to the level of gastric pits and the necks of the pyloric glands. The values varied between 1.5 and 13.5 c.mm. N/20 HCl. No urease was found in the pig's stomach (Linderstrøm-Lang and Holter⁶⁸).

(e) *Arginase*.

The distribution of arginase between the cytoplasm and the nucleus was investigated by Behrens³ in the case of the liver cells. He employed the following method of separation of cytoplasm and nuclei: the finely ground tissue was dried to a powder in the frozen state. It was then suspended in tubes containing varying mixtures of benzene and carbon tetrachloride of different specific gravities. On centrifuging, the particles arranged themselves in layers according to their specific gravity: on the top were found red blood corpuscles, the next layer contained cytoplasm, the following layer contained cytoplasmic particles with nuclei and connective tissue elements, the next layer contained cellular debris of various types, then followed nuclear debris, and the bottom layer contained nuclei only. It was found that cytoplasm and nuclei of liver cells had the same arginase content. This is the first case in which the presence of an enzyme in the cell nucleus has been demonstrated.

(f) *Phosphatases*.

About the relation of these widely distributed enzymes to cell structures only a few facts are known: they play a role in the process of ossification and apparently also in the synthesis of fats in the intestinal mucosa. A classification of phosphatases is given by Folley and Kay²⁶.

1. *Phosphatase in the intestine*. Jeker⁴³ found that in rats treated with monoiodoacetic acid—a specific poison for phosphorylation processes—the absorbed fatty acids were not resynthesized in the epithelial cells covering the villi. If monoiodoacetic acid has been given 1 hour before feeding, the cells remain filled with fatty acid droplets which can be stained by Fischler's²⁵ method (see p. 203) even 6 hours after feeding 3 c.c. olive oil (Plate 2, Fig. 2). This suggests that a phosphatase is involved in the resynthesis of neutral fats in the epithelium covering the villi. (See also reference 74.)

2. *Bone-phosphatase*. The first experiments showing the presence of phosphatase in bone were carried out by Robison^{88,89}. He placed portions of bones of young rats in solutions of barium hexose monophosphate and found a precipitate of barium phosphate after a few hours. This experiment proved the presence of an enzyme in bone that can split phosphoric esters.

(α) *Method of demonstration of phosphatase in bone.* The following method was devised by Robison to demonstrate the presence of this enzyme: Sections of tissue are placed in a solution containing phosphoric esters. By the action of the enzyme, phosphate is liberated which is precipitated as calcium salt in the tissue and stained with AgNO_3 (Kossa's method) or with purpurin or other stains. This allows a fairly accurate localization of the enzyme. For quantitative measurements tissue extracts are used.

(β) *Amount and distribution.* The enzyme is found wherever ossification is taking place or is going to take place. Thus the epiphysal-diaphysal junction of the growing bone has a very high content, as has also the ossifying epiphysis. Slightly lower concentrations were found in the ossifying diaphysis. Hypertrophic cartilage has also a high content of phosphatase, and it was shown by Fell²⁰ and Fell and Robison²¹ that the hypertrophy of the cartilage cell invariably precedes the ossification, e.g. the cells of the cartilaginous embryonic skeleton of the mandible (Meckel's cartilage) never hypertrophy, do not form perichondrial bone, and contain no phosphatase (Fell and Robison²¹⁻²³). The same applies to costal cartilage.

The mandibular bones originate as 'membrane' bones, i.e. from ossification centres developing in the connective tissue, independent of the cartilage. These ossification centres contain phosphatase, as does the ossification centre of the parietal bone, which is another membrane bone. Phosphatase is also found in the growing tooth.

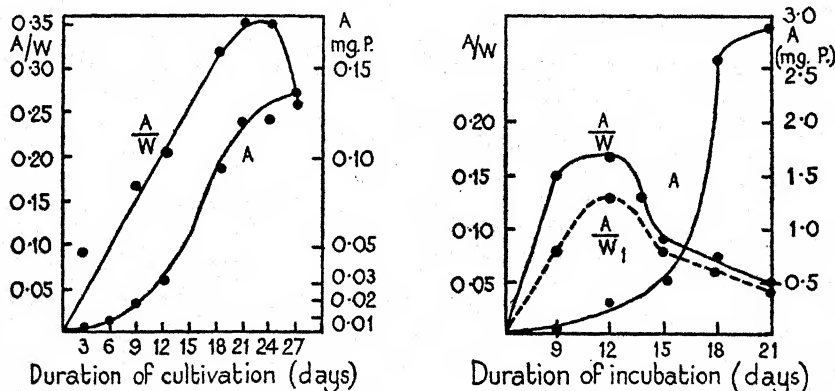
The time when the phosphatase can be first detected in the cartilage coincides exactly with the appearance of hypertrophy of the cartilage cells. This was shown by Fell and Robison^{21, 22} for the developing femur and the palato-quadrate of the fowl *in vivo* and *in vitro*. If the cartilaginous femur rudiment was explanted at an early stage (6-day fowl embryo), it contained no hypertrophic cartilage cells and no phosphatase. After 3 days' cultivation *in vitro*, the first hypertrophic cartilage cells appeared in the middle of the shaft and the first traces of the enzyme could be detected (Text-fig. 12). The bone grown *in vitro* contains more phosphatase per weight than the bone *in vivo*. It is interesting that the enzyme is synthesized *in vitro*, independent of blood circulation, as an intrinsic activity of the cells. Compared with other tissues the following relative phosphatase activities were found by Robison and Soames²¹ in 27-day-old rats (substrate hexose monophosphate; pH = 9):

Ossifying epiphysal cartilage of long bones	100
Costal cartilage (not ossifying)	1
Kidney	47
Liver	5
Muscle	5

The phosphatase activity of human cartilages and bones measured on 1 c.c. of 5 per cent. tissue extract for 18 hours at 30° C. at pH 8.4 (substrate glycerophosphate) was found to be as follows:

		Ester hydrolysed
5½-month-old embryo:	Femur shaft	60 per cent.
	Epiphysial junction	71 "
	Epiphysis (not yet ossified)	0.3 "
	Parietal (membrane bone)	42 "
Infant full term:	Costal cartilage	0 "
	Costochondral junctions ossifying	59 "
Infant 7 months old:	Patella: not yet ossified	0 "
Child 6 years old:	Patella: half containing ossification centre	70 "
	half without ossification centre	0 "

At the temperature and pH of blood the phosphatase present in a growing bone of a rabbit splits enough phosphoric esters in 1 hour to



TEXT-FIG. 12. Phosphatase content of femora of 6-day fowl embryos grown *in vitro* and during normal development (Fell and Robison²¹).

Left. Production of phosphatase during the development *in vitro* of femora from 6-day fowl embryos. A = Phosphatase per femur; given as the amount of hydrolysis (mg. P) of sodium glycerophosphate in 24 hours at 37° and pH 8.5. A/W = Phosphatase per mg. dry weight of femur (weighed after extraction).

Right. Production of phosphatase in the femur of the embryonic fowl during normal development *in ovo*. A = Phosphatase per femur. A/W = Phosphatase per mg. dry weight of femur (weighed after extraction). A/W₁ = Phosphatase per mg. dry weight of femur (calculated on dry weight of corresponding unextracted femur).

precipitate calcium phosphate equal to half the total weight of the bone.

(γ) *The action of the enzyme.* It mainly splits monoesters of orthophosphoric acid. The same type of phosphatase is found in the intestinal mucosa, leucocytes, blood-plasma, and in kidney and mammary gland. Its activity maximum lies at pH 9.4; this distinguishes it from other phosphatases. The activity rises from pH 7 to pH 9 rapidly: e.g. at pH 7.5 the activity is double that at pH 7 and at pH 8 more than fourfold the value at pH 7. It is activated by Mg ions.

The phosphatase of hypertrophic cartilage cells and osteoblasts diffuses from the cytoplasm in the surrounding intercellular spaces.

By its action phosphoric esters are split. This leads to an increase in PO_4^- ions. The importance of this increase was demonstrated by Niven and Robison⁸². They used the tibia and femur of 18 to 19-day rabbit embryos. At this stage hypertrophic cartilage fills the centre of the shaft. The tissue was immersed for 16 hours at 37°C . and pH 7.4 in solutions containing 8 mg. Ca and 4 mg. inorganic P in 100 c.c. with or without the addition of organic P.

The results were that immersion in a solution without phosphoric esters led to the deposition of only small amounts of calcium phosphates, whereas the addition of 3 mg. organic P as glycerophosphoric ester to this solution brought about an intense calcification of the hypertrophied cartilage and the osteoblastic tissue (Plate 2, Figs. 3 and 4). The conclusion drawn from these experiments is that two processes are involved in the process of calcification: (1) the action of the phosphatase leading to a supersaturation of the intercellular fluid and matrix with bone salts, and (2) a mechanism leading to the deposition of bone salts. This may be due to local changes in pH or other factors, e.g. vitamin D. Very little is known about this second mechanism. Treatment with fat solvents impairs the second process, but not the first. Desiccation and very low concentrations of KCN act similarly; glucose also inhibits the second mechanism.

(δ) *Phosphatase in diseases of the skeletal system.* In rickets a broad irregular band of hypertrophic cartilage and osteoid tissue is found at the epiphysial junction. Policard, Péhu, Roche, and Boucomont⁸⁵ give the following values of phosphatase activity for 100 mg. tissue of the proximal part of the human tibia, expressed in mg. P liberated in 24 hours at 37°C . and pH 8.6 from 5 c.mm. M/10 sodium glycerophosphate.

	<i>Hyaline cartilage</i>	<i>Hypertrophic cartilage</i>	<i>Ossification zone (or osteoid)</i>	<i>Bone of diaphysis</i>
Normal . . .	0	1.26	1.63	0.75
Rickety . . .	0	1.90	2.65	1.48

This shows clearly that a higher amount of phosphatase is present in ricketic bones, but calcium and phosphate cannot be deposited owing to the deficiency of the fat-soluble vitamin D. In this regard it is of great interest to note that fat solvents impair the deposition of bone salts in normal slices of bone (see above), and that normal cartilage cells and osteoblasts always contain small granules which stain with Sudan and reduce osmic acid.

Plate 2, Figs. 5 and 6, illustrate the high content of phosphatase of the uncalcified hypertrophic cartilage and of the incompletely ossified bone trabeculae of the epiphysial-diaphysial junction in rickets. One radius had been immersed for 16 hours in a solution of sodium phosphate

(Plate 2, Fig. 5), the other in a solution of calcium hexose monophosphate (Plate 2, Fig. 6). By the action of the phosphatase intense calcification took place *in vitro* both in hypertrophic cartilage and osteoid tissue, which could not take place *in vivo* owing to the absence of other factors.

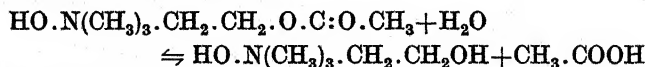
Huggins⁴⁰⁻⁴² has shown that when bladder epithelium of the dog is transplanted to the sheath of the abdominal rectus and survives, after 18 days the surrounding connective tissue will form bone. The deposit of heterotopic bone goes parallel with the formation of phosphatase. The following figures for enzyme activity were found:

Controls		After transplantation of bladder mucosa in the rectus sheaths
Rectus sheaths . . .	0	Rectus sheaths 0.1
Bladder mucosa . . .	0.2	Rectus sheath with bladder epithelium and ossification . . 0.20

So far only the ester-splitting action of the bone phosphatase was dealt with, but deposition of calcium phosphate (probably as calcium carbonate apatite) proceeds in the growing bone side by side with resorption of the bone matrix (by osteoclasts). Very little is known whether and at what stage of the resorption of bone soluble phosphoric esters are resynthesized by this enzyme; but Martland and Robison^{73, 73a} and others could show *in vitro* that phosphatase can resynthesize phosphoric esters. It is known that hyperparathyroidism leads to the appearance of large numbers of osteoclasts and the removal of the bone salts. Further investigation is required to establish whether a synthetic 'osteoclastic' action of the phosphatase takes part in the resorption of bone.

(g) Cholinesterase.

This enzyme catalyses the hydrolysis of acetylcholine to choline and acetic acid (and vice versa) thus:



Acetylcholine is of biological significance as a transmitter substance, that is, as a chemical messenger released at nerve endings. First shown to act as the 'vagus substance' in the frog's heart by O. Loewi, it is now generally believed to transmit impulses: (a) at all ganglionic synapses of the autonomic nervous system, i.e. in both sympathetic and parasympathetic ganglia; (b) at many postganglionic autonomic endings, chiefly in parasympathetic fibres; (c) at the motor endings in skeletal muscle. Nerve fibres which act by releasing acetylcholine are called 'cholinergic' (Dale). The list of cholinergic fibres given above refers

only to vertebrate nerves, but occurrence of cholinergic nerves in invertebrates, e.g. in annelid worms and in crustacea, has been shown to be likely.

Cholinesterase is responsible for the inactivation of the transmitter after it has done its work. It is obvious that a substance which has such powerful effects must be prevented from spreading and stimulating distant excitable structures. In addition, the transmitter must be prevented from stimulating for too long a period.

The distribution of the enzyme, as studied in the mammal, corresponds to these functions:

(a) The enzyme is present almost everywhere, in cells as well as in tissue fluids; for biochemical studies it is usually obtained from blood plasma.

(b) The enzyme is concentrated wherever cholinergic fibres innervate an excitable structure with a short refractory period, e.g. in striated muscle or in the sympathetic ganglion. In the case of striated muscle the distribution of cholinesterase in the sartorius of the frog (*Rana esculenta*) has been studied. This muscle was chosen because the distribution of nerve endings in the muscle has been well studied. The muscle is known to be free of nerve endings in its pelvic end. A careful study of the distribution of nerve endings was recently made by Pézard and May⁸³. They applied a modification of Ramon y Cajal's silver method and made serial sections of the muscle and counted the nerve endings. They found that the pelvic end—about one-eighth of the length of the muscle—was completely free of nerve endings, the pelvic fifth contained only very few endings, but the adjoining fifth of the muscle was extremely rich in endings. The distribution of cholinesterase was determined as follows (Marnay and Nachmansohn⁷²): the muscle was divided into five parts, the tissues were finely ground, and the esterase activities of the different portions determined. The extracts were suspended in a bicarbonate solution and acetylcholine was added. The acetic acid formed in the hydrolysis of acetylcholine liberated CO₂ from the solution; the rate of CO₂ formation was determined manometrically. The activities are expressed as QChE, i.e. as mg. acetylcholine chloride hydrolysed by 100 mg. of tissue in 60 minutes. For the two adjoining portions the following QChE values were found:

Pelvic end: 0.135 Second fifth: 0.795.

In order to know the relative concentration of the enzyme in or around the nerve endings, it would be necessary to have data about the volume of the motor endings, but it is obvious that the concentration of the enzyme at or around the endings must be considerable. (The authors give arguments which show that the increased activity in the portions containing the endings cannot be due to the presence of the nerve fibres.) The localization of cholinesterase has also been studied in

mammalian muscle (Couteaux and Nachmansohn⁸). The guinea-pig's gastrocnemius was frozen and sections were used for histological examination and determination of esterase activity. The distribution of the terminal branches of the nerve in the muscle makes it possible to obtain end-plate-free sections and sections rich in end-plates. The latter sections have the higher esterase activity.

In the sympathetic chain of the cat, the QChE of the superior cervical ganglion was 45 to 60, that of the afferent fibres in the sympathetic nerve about 5. Similar differences occur in the abdominal chain of the lobster where the ganglia contain more than twice the amount of activity of the fibres⁷⁸⁻⁸⁰.

The electric organ of the ray, *Torpedo marmorata*, is generally considered as a collection of modified motor end-plates. Determination of the cholinesterase activity of the organ showed that the organ has a very high cholinesterase activity (Marnay⁷¹). It was this finding which led to an analysis of the nerve supply to the electric organ by Feldberg, Fessard, and Nachmansohn¹⁸ in which the cholinergic nature of the innervation was established: if the organ is made to discharge by electrical stimulation of its nerve, acetylcholine can be discovered in the perfusion fluid, and close-range arterial injection of acetylcholine causes a discharge of the organ. The esterase activity in the electric organs of different species varies. This is shown in the following table⁷⁹.

Species	QChE
<i>Raja</i> . . .	3-10
<i>Gymnotus</i> . . .	90-150
<i>Torpedo</i> . . .	150-300

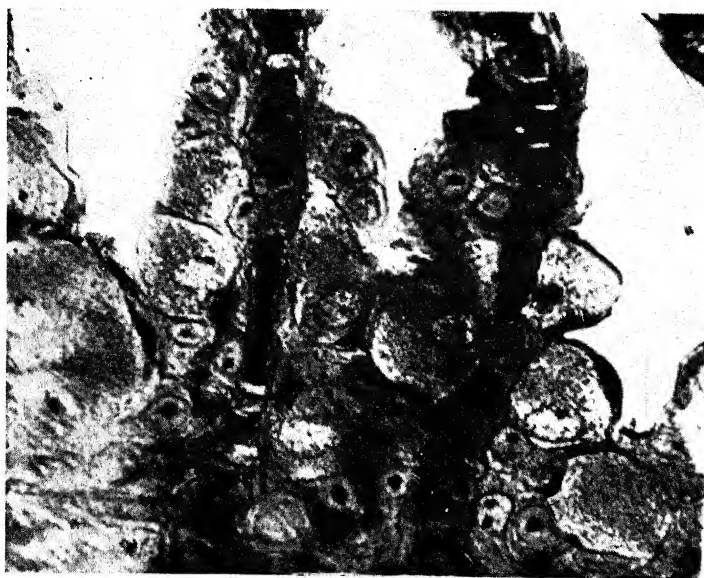
For the central nervous system there exists up to the present no clear-cut evidence in favour of cholinergic transmission, although there exist some indications in favour of the existence of a cholinergic mechanism. The esterase content of the mammalian brain has recently been studied. It was found that the esterase is very unevenly distributed. The white matter is poor in esterase activity; more of the enzyme is found in the grey matter, but the activities differ greatly in different parts. The following table gives data for cholinesterase activities as found in the ox brain.

The reason for the uneven distribution of the enzyme in the grey matter is unknown. That the enzyme is somehow connected with the function of the central nervous system is made likely by the study of it during development. The esterase activity of the chick's brain is already high at the time of hatching. At that time most of the synapses in the brain must already be functioning. In mammals characteristic differences are found in different species: in the guinea-pig, an animal which is already well developed at the time of birth, the rise in esterase

	<i>QChE</i>
White matter.	0.2-0.3
Cortex	2-3
Caudate nucleus	40-43
Lenticular nucleus (putamen)	68-70
Cerebellum	2.5-4
Thalamus	2.5-5
Corp. quadrigem. ant.	9.5-13
" " post.	4

activity during development occurs chiefly before birth; in animals like the rat, the cat and the rabbit, which are born in a much less mature stage, the final figures for esterase activity in the brain are only attained some time after birth. In sheep embryos the esterase activity in the spinal cord is high at a time when reflex muscular movements occur (60 to 80 days) (Nachmansohn⁸⁰). The fact that high cholinesterase activity occurs at synapses raises the question: Where is the enzyme localized? Is it present within the endings or in the surroundings of the endings? No direct evidence on this point is available. If the motor nerve to a skeletal muscle is cut, degeneration of the nerve fibres in the muscle occurs, but there is no decrease in the esterase activity. In fact, an increase of esterase concentration is observed after degeneration. This is explained by the reduction of the volume of the muscle during degeneration, the total amount of enzyme remaining approximately unchanged. Using frozen sections it can be shown that the high esterase concentration in the region of the end-plates is maintained after degeneration. On the other hand, in the superior cervical ganglion of the cat the esterase activity decreases during degeneration of the pre-ganglionic fibres (v. Bruecke⁷), but the final value for the activity is still 4 to 5 times above that for the fibres. From these experiments Couteaux and Nachmansohn conclude that in the motor end-plates the enzyme is localized not in the nerve ending but in the sarcoplasmic part of the end-plate, whereas in the ganglion the enzyme occurs in the endings as well as in their surroundings. Boell and Nachmansohn^{5a} have recently shown in the giant axon of the squid that the esterase is concentrated in or around the sheath; the axoplasm has a much lower activity.

Glick²⁹ has studied the distribution of cholinesterase in the gastric mucosa by the methods developed in Linderström-Lang's laboratory. He finds that the cells of the surface epithelium are particularly rich in the enzyme; the activity is least in the muscularis. The data given for the activity in the surface epithelium correspond to a *QChE* of about 20, that is, a very high activity. The distribution of the enzyme in the mucosa suggests that the enzyme fulfils some function not connected with the destruction of a transmitter substance.



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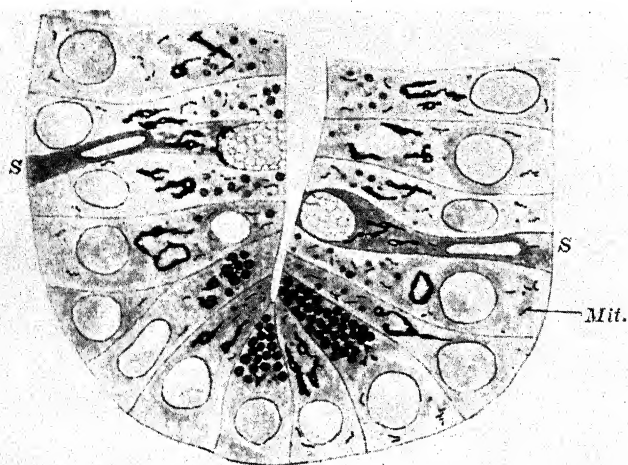


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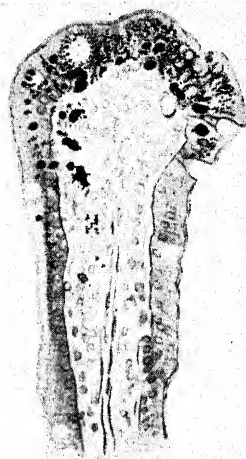


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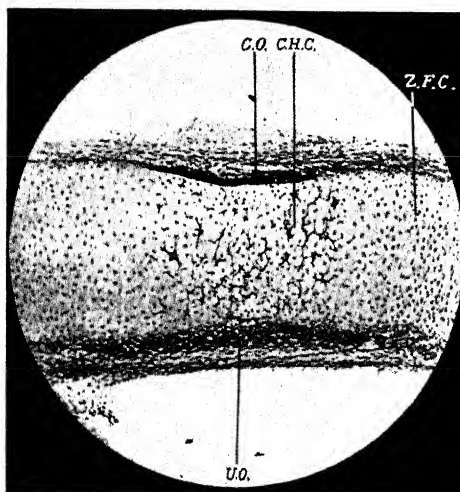
FIG. 1. Eosinophil cells from the gill leaflets of the eel. FIGS. 2-5. Action of lipase in intestinal cells. *For description see p. 225.*



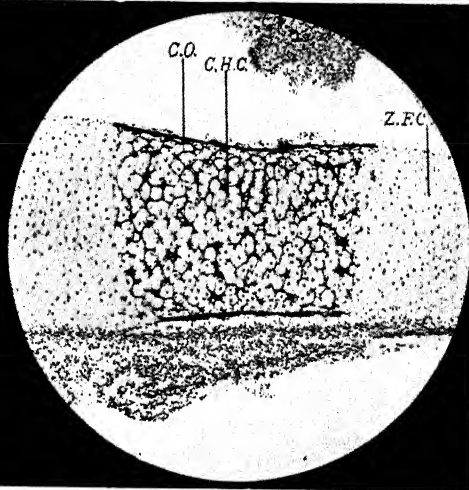
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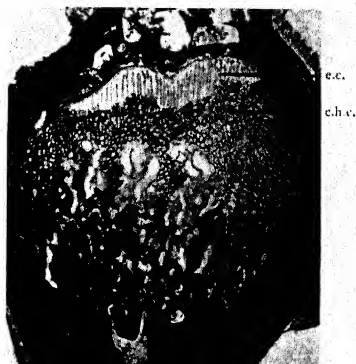
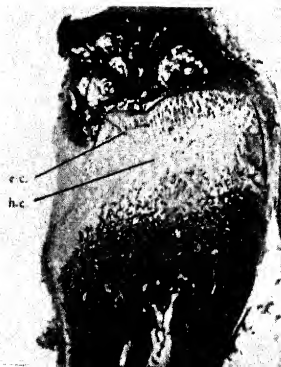
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DESCRIPTION OF PLATES

PLATE 1

FIG. 1. Microphotograph ($\times 800$) of eosinophil secretory cells from the gill leaflets of the eel. (After Keys and Willmer⁵².)

FIG. 2. Villus of small intestine of rat, 30 minutes after feeding 3 c.c. olive oil, showing fatty acid in the epithelial cells as black droplets (Fischler's method). (Jeker⁴³.)

FIG. 3. Villus of small intestine of rat 6 hours after feeding 3 c.c. olive oil, stained as in Fig. 2. The epithelial cells are filled with droplets which remain unstained but are Sudan positive. The lipase has resynthesized neutral fats from fatty acids. Only a few fatty acid droplets (black) remain in the cells. (Jeker⁴³.)

FIG. 4. High-power drawing of an epithelial cell at the stage of Fig. 2.

FIG. 5. High-power drawing of an epithelial cell at the stage of Fig. 3.

PLATE 2

FIG. 1. Crypt of Lieberkühn ($\times 900$) showing three Paneth cells with their specific granules at the bottom of the crypt, also two goblet cells (S). Mitochondria (Mit.) and Golgi apparatus (black threads) in all cells. (van Weel⁹⁷.)

FIG. 2. Villus of small intestine of rat, 7 hours after a dose of monoiodoacetic acid which was followed 1 hour later by 3 c.c. olive oil, stained by Fischler's method. The black droplets of fatty acid are still found in the epithelium; some have diffused into the stroma. Synthesis of neutral fats has been blocked. Compare with Plate 1, Fig. 3. (Jeker⁴³.)

FIG. 3. Longitudinal section of a tibia of an 18-day rabbit embryo. Calcification black by treatment with AgNO_3 , nuclei counterstained with Carmalum. $\times 55$. The diaphysis is composed of hypertrophic cartilage, partly calcified (C.H.C.), and a small layer of periosteal bone (C.O.) on one side. At the other side the periosteum has not yet formed bone (U.O.). Z.F.C. Zone of flattened cartilage cells. $\times 55$. (Niven and Robison⁸².)

FIG. 4. The other tibia of the same embryo, immersed for 22 hours at 37°C . (pH 7.4) in a solution containing 8 mg. Ca, 4 mg. inorganic P, and 3 mg. organic P (as glycerophosphoric ester) in 100 c.c. Note heavy disposition of calcium salt in the matrix of the hypertrophic cartilage and in the periosteal layer by the action of the phosphatase. Stained as in Fig. 3. $\times 55$. (Niven and Robison⁸².)

FIG. 5. Epiphysal-diaphysal junction of the radius of a ricketic rat, after 16 hours' immersion in 0.1 M sodium phosphate at 37°C . Note the broad band of hypertrophic cartilage (h.c.) that has remained uncalcified and also the incomplete ossification of the osteoid trabeculae; e.c. small cell cartilage. (Robison⁹⁰.)

FIG. 6. The same region of the other radius of the same rat, after 16 hours' immersion in 0.1 M. Calcium hexose-monophosphate, as the only source of phosphate. By the action of the phosphatase the hypertrophic cartilage and the osteoid trabeculae are heavily calcified. The small cell cartilage (e.c.) remains uncalcified. Calcified hypertrophic cartilage (c.h.c.). (Robison⁹⁰.)

CHAPTER VIII

PATHOLOGICAL ASPECTS OF CYTOLOGY

By R. J. LUDFORD

i. INTRODUCTION

THE structure of cells varies with their state of functional activity. The range of variation in cellular structure is well illustrated by gland cells in various phases of activity. Precise morphological differences characterize 'resting' and dividing gland cells, and those engaged in the elaboration of their specific secretions. Such changes are the expressions of the normal range of physiological functioning. Other modifications of cell structure accompany aberrations of functioning, are the result of alterations in the humoral environment, or are consequent upon infections. These latter constitute the pathological modifications of cellular structure.

ii. REGRESSIVE CHANGES

Cellular degeneration is the most frequent result of pathological conditions. The morphological changes which occur in degenerating cells are most conveniently studied in living tissue cultures, especially in those prepared with fluid culture media. Under such conditions well-spread cells can be obtained on the surface of cover-glasses. They are best examined by washing off the culture medium and mounting in a saline solution. Such preparations are ideal for examination by dark-ground illumination, which permits of the most detailed observation of changes in protoplasmic structure.

When a cell from an actively growing culture of mammalian fibroblasts is examined in this way (Plate 4, Fig. 1) the only nuclear structures clearly distinguishable are one or more nucleoli and the nuclear membrane; but in the cytoplasm the mitochondria are conspicuous objects. There are also usually to be seen a few minute cytoplasmic granules which stain readily with neutral red, and a few small, highly refractile fat droplets. What is generally described as the 'ground cytoplasm' is optically empty. Centrioles are not distinguishable: nor is the Golgi apparatus. This does not necessarily imply that the Golgi apparatus as demonstrated by the osmic or silver impregnation techniques is an artifact. It is probable that its refractive index does not differ sufficiently from that of the surrounding cytoplasm to render it visible.

When cultures are prepared and simply incubated, without receiving any further treatment, conditions soon become unfavourable for growth,

owing to the accumulation of toxic metabolic products and depletion of essential nutritive requirements. There is thus initiated a progressive series of cellular changes, often continuing slowly for several weeks, before terminating in the death of the cells. It will be convenient to discuss separately the changes occurring in (a) the mitochondria, (b) the ground cytoplasm, (c) the sphere, (d) the Golgi apparatus, and (e) the nucleus.

a. The Mitochondria.

The mitochondria are particularly sensitive to pathological conditions. In actively growing cells they tend to be mostly filamentous and spread throughout the greater part of the cytoplasm. With the advent of adverse conditions they begin to collect around the nucleus, usually becoming heaped up around the central body. Filaments break up into granules, and some swell and become vesicular, as seen in Plate 1, Fig. 1. In advanced stages of degeneration the mitochondria are reduced to fine granules, scattered amongst other cytoplasmic granules, with usually a few short rod-like forms at the extreme periphery of the cell. They still retain their specificity for the vital dyestuffs, Janus green and Janus black, which renders their identification possible. Finally, they appear to disintegrate completely.

Under certain pathological conditions *in vivo* mitochondria have been reported to undergo fatty changes. In a fat-cell tumour (lipo-sarcoma) of the guinea-pig Murray¹⁴⁸ described the mitochondria swelling up and becoming converted into fat droplets. In degenerating tumour cells the fragmented mitochondria also appear to give rise to fat droplets (Ludford¹¹⁹). Scott¹⁸⁰ found that the filamentous mitochondria of acinar cells of the pancreas became granular in phosphorus poisoning. The granules fused together and gave rise to fat droplets. According to Kater⁸¹ fat droplets arise within the mitochondria of liver cells. As such fat droplets enlarge they are surrounded by an incomplete peripheral film of mitochondrial substance. Duthie⁴⁸, however, disagrees with this. He describes the mitochondria of degenerating liver cells as becoming enspherulated and enlarged, then vacuolated; and finally disappearing without forming fat. A similar process was described by Ostrouch¹⁵⁹ as being brought about by the action of ethyl alcohol on epithelial cells of the stomach. The mitochondria became granular, lost their polarized distribution above and below the nucleus, enlarged and then disappeared as the cytoplasm became vacuolated. According to Grynfeltt and Lafont⁷⁰ the mitochondria of liver cells became granular with sulphonal poisoning and were displaced towards the periphery of the cells.

Although mitochondria of most cells are particularly sensitive to pathological conditions, yet those of nerve cells seem to be exceptional.

Cowdry³⁰ cites as examples of their stability under pathological condition the retention of their normal appearance in nerve cells injured by the viruses of poliomyelitis (McCann¹⁴⁴), and herpetic encephalitis (Cowdry and Nicholson³⁸).

b. The Ground Cytoplasm.

Although the ground cytoplasm of healthy fibroblasts growing in tissue cultures is optically structureless, it often contains a few granules which stain with neutral red, and other basic dyes such as methylene blue and cresyl blue. Lewis⁸⁷ calls all such neutral red staining particles 'degeneration granules'. Carrel and Ebeling¹⁴, however, consider a juxta-nuclear group of neutral red granules to constitute a 'segregation apparatus', which enlarges by increase in number and size of the constituent granules with increased rate of cell growth. Rumjantzew¹⁷⁸ also supported this conception of a segregation apparatus. Both Lewis⁹¹ and Ludford¹²⁸, however, found few or no neutral red granules in young actively growing fibroblasts, but observed their accumulation as cultures deteriorated. They accumulate around the sphere, spreading out towards the periphery of the cell. Lewis⁹¹ observed the formation of vacuoles about these granules: but they may fill almost the whole of the cytoplasm without any large vacuoles being formed, as can be seen in the binucleate cell of Plate 1, Fig. 4. It is in such cells as this that one often sees a clear reticulate area of cytoplasm which bears a striking resemblance to the Golgi apparatus of fixed tissues.

In addition to the formation of degeneration granules, there may also be considerable vacuolation of the cytoplasm (Plate 1, Fig. 3), and formation of fat droplets. All three processes may occur concurrently in the same cell: but neighbouring cells in the same culture may exhibit very different degenerative changes. Lewis suggested that the degeneration granules and associated vacuoles are the accumulations of the waste products of autolysis. He found that the same sort of cytoplasmic granules and vacuoles were found when cells degenerate in the body of dead animals as when they are incubated in saline *in vitro*. There was, however, considerable difference in the rate at which different cells degenerate and die. Thus macrophages survive for about 10 days and brain cells for less than an hour (Lewis and McCoy⁹⁹). Experiments on cytoplasmic viscosity led von Möllendorff¹⁴⁵ to the conclusion that vacuole formation is a cellular defence mechanism against substances injurious to cells. Substances such as potassium chloride and potassium acetate, which lower cytoplasmic viscosity and increase the rate of movement of cytoplasmic granules, subsequently bring about vacuole formation at the expense of the granules, thereby restoring the normal viscosity. The acid vital dyestuffs, colloidal particles, and fat are alike segregated in vacuoles and so prevented from interfering with cellular

metabolism. The basic vital dyestuff neutral red when applied to living cells first colours the cytoplasm diffusely, but is soon flocculated on the surface of granules and vacuoles. During the phase of diffuse staining mitosis is inhibited, but can proceed after the dyestuff has been segregated, if excess of the dyestuff is washed away. Mitosis can proceed quite normally in cells containing numerous fat droplets. Nagel¹⁵² has drawn attention to the fact that cells containing many vacuoles which stain vitally with basic dyestuffs contain few fat droplets, and vice versa. Fat droplets also tend to collect around the sphere like the 'degeneration granules' and vacuoles, Plate 1, Fig. 2. The origin of the fat appearing in cells *in vitro* has been the subject of much discussion in connexion with the problem as to the source of fat in cases of fatty degeneration of tissues. With cells growing *in vitro* the extent to which they become laden with fat droplets depends to a large extent upon the medium (Lambert⁸⁴). The addition of yolk to the culture medium results in the appearance of fat in the cells (M. R. Lewis⁸⁶). Rat and mouse cells develop more fat droplets in mouse serum than in the less fatty rat serum (Ludford¹²⁵). The extensive accumulation of fat in degenerating cells described by Horning and Richardson⁷⁷ is a characteristic feature of chick tissues grown in fowl plasma, and contrasts strikingly with the degenerative process occurring in rat cells grown in rat serum in which there is little or no fat formation. It is clear, therefore, that fat may be of extracellular origin and be segregated in droplets in the same way as are other foreign substances. Dible and his collaborators have adduced evidence in a series of researches that the accumulation of fat droplets in cells of the liver, heart, and kidney under various pathological conditions is always associated with an increase in the fat content of these organs, and that this excess results from infiltration of the cells. Furthermore, this fatty infiltration is dependent upon adequate fat in the animal's storage deposits (see Dible and Gerrard⁴⁵; Dible and Hay⁴⁶). Other investigators have contended that fat droplets originate from the disorganization of the cytoplasmic colloids (phanerosis). There has been much discussion as to whether proteins and lipides exist in the cytoplasm in a state of physical or chemical combination. From his critical survey of the literature Kiesel⁸² concludes that the two types of substances form adsorption complexes rather than chemical compounds. Pollack's¹⁶⁷ experiments in which he precipitated protein from aqueous solutions of dead *Amoebae* with picric acid, but on injecting the same solution into the living organisms obtained no precipitate, raises doubts as to whether proteins, as such, exist in living cells. According to Nadson¹⁴⁹, Nadson and Meisl¹⁵⁰, and Nadson and Rochlin¹⁵¹, in plant cells separation of lipides and proteins is brought about by irradiation and chloroform poisoning, and results in the formation of fat droplets, proteins being irreversibly coagulated at the same time.

Cells in tissue cultures exhibiting early degenerative changes are able to recover if they are transplanted to fresh culture media. Contact of fibroblasts with cultures of monocytes and macrophages also has a rejuvenating effect, leading to the disappearance of fat droplets and cytoplasmic granules, and stimulation of growth (Carrel¹²; Ludford¹³²). The final changes of cell degeneration *in vitro* involve what remains of the ground cytoplasm, which becomes filled with very fine granules, best seen by dark-ground illumination. If the cells have been vitally stained the colour diffuses from the mitochondria and from the 'degeneration granules' and vacuoles. The nuclear membrane becomes more clearly defined, the nucleolus is coloured, and the fine granulation spreads throughout the nucleus. Any vital dye present stains the whole cell diffusely. The cell colloids are now irreversibly coagulated.

c. The Sphere or Central Body.

In healthy actively growing cells *in vitro* the central body is not a clearly defined structure, although the mitochondria tend to collect at one side of the nucleus where in fixed and stained preparations the centrioles are distinguishable. Mitochondria move backwards and forwards from this juxta-nuclear area to the cell periphery. When 'degeneration granules' make their appearance they accumulate in this area. Later in the midst of the group of granules a clear area appears, and it is in the centre of this that the centrioles are demonstrable after fixation and staining. Filamentous mitochondria usually become radially arranged around it. In some cells this central area undergoes a remarkable hypertrophy, resulting in a so-called giant centrosphere, which may be larger than the nucleus. According to Lewis⁸⁸, in its complete form it consists of a centriole contained within a medullary zone which stains deeper than an external cortical zone, and from which it is separated by a thin membrane. Lewis⁹² has observed giant centrospheres only in cultures of mesenchyme, mesothelium, and endothelium.

The origin of the substance forming these large bodies remains obscure. Lewis⁸⁸ suggests that the clear area around the centriole results from the accumulation of some of the cytoplasm flowing centripetally towards it. This streaming would account for the radial arrangement of the mitochondria. The material thus accumulated probably acquires a more solid consistency, thus preventing mitochondria and 'degeneration granules' from penetrating into it, and pushing them outwards as it enlarges. These giant central bodies are of interest owing to their similarity to certain inclusions that occur in some malignant cells, and which at the time of their discovery were mistaken for the causative organisms of malignant disease. Of these inclusion bodies it now seems quite clear that some were enlarged central bodies, and other products of abortive secretory activity (see Ludford¹⁰⁹).

d. The Golgi Apparatus.

Reference has already been made to the fact that a reticulate area resembling the Golgi apparatus is visible in some degenerating cells, Plate 1, Fig. 4. A similar colourless network can also be demonstrated under certain conditions in cells, the cytoplasm of which has been diffusely stained with methylene blue (Ludford¹²⁷). Under no other condition has any structure resembling the Golgi apparatus been seen in living cells in tissue cultures. In fixed cultures suitably impregnated by either the osmic or silver techniques; it has been demonstrated in a variety of normal and malignant cells (Ludford¹¹⁰; Zweibaum and Elkner¹⁹⁹; Vázquez López¹⁹²; Saguchi¹⁷⁹; Richardson¹⁷⁰; Macdougald and Gatenby¹³⁹; and Macdougald^{137,138}). Apart from Zweibaum and Elkner, who accepted the Parat conception of a zone of Golgi consisting of 'chondriome actif' and 'vacuome', the other investigators are in agreement that the Golgi apparatus is a cytoplasmic structure independent of neutral red staining vacuoles. There is also general agreement that the Golgi apparatus breaks up into granules in degenerating cells. Ludford's observations led to the conclusion that the fragmented particles of the Golgi apparatus gave rise to fat droplets. Richardson confirmed this observation, as did also Macdougald, and Macdougald and Gatenby. In his latest paper, however, Macdougald¹³⁸ denied that the brown droplets resulting from the fragmentation of the Golgi apparatus in osmic preparations were fat, since they did not stain with Sudan III.

Fragmentation of the Golgi apparatus has been described under a variety of pathological conditions, during autolysis of normal and malignant cells (Cajal¹¹; Ludford¹¹⁹), in kidney tubules in experimental nephritis (Pappenheimer¹⁶⁰), in nerve cells following section of axones (Penfield¹⁶³), in cells subjected to the action of poisons, and following irradiation. A considerable enlargement of the apparatus with thickening of its strands, unaccompanied by secretory activity, occurs in acinar cells of the pancreas after repeated injections of neutral red (Ludford¹¹⁸). Such cells present the appearance of having had their secretory mechanism blocked by the accumulation of the dyestuff.

The sensitivity of the Golgi apparatus to injury probably varies in different types of cells, as is the case with mitochondria. It is of interest to note that in studying the effect of phosphorus poisoning and irradiation on the spermatogenesis of *Abraxas*, Gatenby^{59,60} found that the Golgi apparatus was the most sensitive of the cell organs.

e. The Nucleus.

The degenerative changes occurring in the nuclei of cells growing *in vitro* have not received the same attention as the cytoplasmic ones. As already described, the cytoplasm can be studied by the aid of vital staining, and finer changes in its structure revealed by dark-ground

illumination. Neither of these techniques is suitable for the study of the nucleus. Vital dyes do not penetrate the nucleus until the cell has reached an advanced stage of degeneration or is subjected to special physiological conditions (see Ludford¹²²), and dark-ground illumination only reveals clearly the nucleolus and nothing of the chromatin.

Nucleolar extrusions into the cytoplasm have been described in cultures of endothelium (Lewis⁹⁰) and fibroblasts (Ludford¹⁰⁷). As the same process has been reported in many different types of cells, under varying physiological conditions *in vivo*, it is doubtful whether its occurrence in tissue cultures is indicative of pathological change. The formation of nuclear buds which become nipped off and enclosed within cytoplasmic vacuoles is an early indication of abnormality. They remain for a time in the cytoplasm, resembling somewhat the secondary nuclei of Hymenopteran oöcytes, but ultimately disintegrate. This nuclear budding may represent the initial phase of nuclear fragmentation, which results in large nuclei becoming broken up into a number of smaller ones (Macklin¹⁴⁰). This is commonly seen in malignant cells *in vivo* (Howard and Schultz⁷⁸), and would seem to be directed towards increasing the area of the nuclear-cytoplasmic interface, which otherwise becomes progressively reduced, in proportion to the volume, as cells enlarge.

The disintegration of the nuclear structure may occur with or without the formation of acidophil deposits. In the latter case the chromatin coalesces and forms irregular-shaped masses applied to the nuclear membrane (karyorrhexis), or it appears to liquefy (karyolysis). According to Roskin¹⁷⁴, in degenerating tumour cells either chromatin accumulates so as to form hyperchromatinic nuclei, or else it is extruded into the cytoplasm and the nuclei become hypochromatinic.

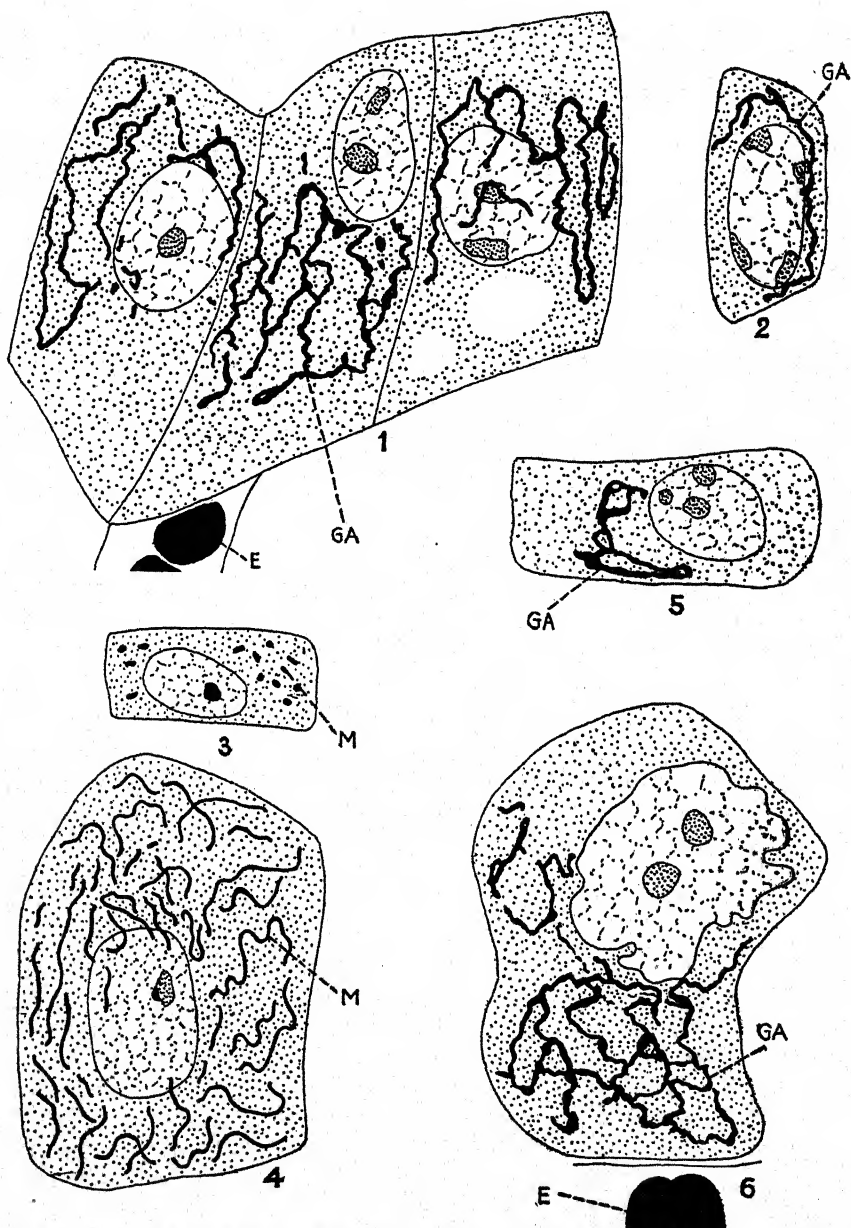
Cell enlargement with a relative increase in size of the nucleus frequently precedes nuclear disintegration. It occurs after irradiation in tumour cells (Mottram¹⁴⁶) and is followed by the disintegration of chromatin (thymonucleic acid protein complexes) with the formation of an achromatinic substance, which forms nucleolar-like bodies (Ludford¹¹⁹). In the liver and kidney of vitally stained animals, and after poisoning with yperite and Lewisite, Wermel and Ignatjewa¹⁹⁵ have described considerable enlargement of some cells. Their nuclei contained relatively large masses of nucleolar material. The formation of achromatinic substances within nuclei has received much attention in connexion with the intranuclear bodies, which are characteristic of some virus diseases, and will be further discussed in a later section.

iii. CELLULAR HYPERACTIVITY

✓ The cellular processes with which we have so far been concerned have been regressive in character. Tissue lesions, however, are invariably followed by intensified cellular activity directed towards the repair of

injury or functional compensation. Thus, destruction of muscle fibres by infiltrative tumour growth is often followed by growth of sarcoplasm at certain parts of the fibres, and a local increase in the number of nuclei. There is also an increase in the number of mitochondria and Golgi bodies (Ludford¹⁰⁸). With compensatory hypertrophy of the kidney, Hirsch⁷⁵ and others have described increased numbers of mitochondria. Nicholson¹⁵³ observed the same in compensatory hypertrophy of the thyroid gland. In malignant glandular cells that retain their capacity to elaborate secretion, the mitochondria are numerous or elongated and the Golgi apparatus enlarged as in gland cells engaged in the elaboration of their specific secretion. When tumours of this type are irradiated, there follows a period during which mitosis is inhibited, but the cells increase in size, and present the cytological characteristics of intensified secretory activity. The abortive acrosome formation induced by irradiation in spermatocytes of *Cavia* and *Abraxas* probably also results from a similar persistence of functional activity of the cytoplasmic organs during the period when the spermatocyte divisions are inhibited (Gatenby and Wigoder⁶¹; Gatenby, Mukerji, and Wigoder⁶²).

One of the best examples of hyperactivity of cellular function is afforded by the thyroid gland in exophthalmic goitre (Graves' disease). Goetsch⁶⁵ observed an increase in the number and length of the mitochondria in toxic adenomata of the thyroid associated with clinical symptoms of hyperthyroidism; and Bolt¹⁰ confirmed these observations. Cramer and Ludford⁴² investigated the cytological changes in the thyroid gland under various physiological conditions, and found that the phase of intense secretory activity was characterized by long filamentous mitochondria and an enlarged Golgi apparatus. In a further investigation they studied the cytology of the thyroid in a case of exophthalmic goitre in the mouse, and three human cases of the disease (Ludford and Cramer¹³⁴). The most distinctive cytological feature was the extreme degree of enlargement of the Golgi apparatus. Text-fig. 2 shows a cell with the type of Golgi apparatus characteristic of the resting phase, while Text-fig. 1 represents three cells exhibiting the extreme degree of enlargement of the apparatus, from the mouse case. Mitochondria were numerous in the enlarged cells and were filamentous. In many of the cells they were rounded and swollen to form plastid-like bodies. Text-figs. 5 and 6 show the variation in size of the apparatus in the human cases, and Text-figs. 3 and 4 the difference in the mitochondria in a relatively inactive cell and in an actively secreting cell. It is of interest to observe that the human cases had been treated with iodine preliminarily to operative removal of the glands. In both the human and mouse glands reversal in polarity of the Golgi apparatus was observed (Text-figs. 1 and 6), so that it was suggested that this indicated the direct discharge of the specific hormone into the blood-stream. ✓



TEXT-FIGS. 1-6. Cellular hyperactivity in thyroid gland cells in exophthalmic goitre.

Fig. 1. Three enlarged cells showing hypertrophy of the Golgi apparatus, with reversal of polarity in the middle cell. Fig. 2. Relatively normal cell with type of Golgi apparatus characteristic of the 'resting' phase. Fig. 3. Mitochondria in an inactive cell. Fig. 4. Mitochondria in an actively secreting cell. Fig. 5. Golgi apparatus of cell in early stage of secretory activity. Fig. 6. Actively secreting cell with hypertrophied Golgi apparatus exhibiting reversed polarity.

E, erythrocyte in capillary; GA, Golgi apparatus; M, mitochondria.

Figs. 1 and 2 from a case of exophthalmic goitre in a mouse. Figs. 3-6 from human cases. After Ludford and Cramer¹²⁴.

Okkels¹⁵⁶ has examined a large number of resected human thyroid glands. Forty-four of these were from patients with pronounced Graves' disease. These all exhibited enlargement of the Golgi apparatus. Another fourteen cases were diagnosed histologically as colloid goitre with islands of epithelial hyperplasia. Ten of these were clinically thyrotoxic cases and showed enlargement of the Golgi apparatus; but of thirty-nine simple colloid goitres, only eight possessed a few scattered cell clusters exhibiting enlargement of the Golgi apparatus. Twenty-five of Okkels's cases of Graves' disease had received iodine treatment, but although in many there resulted such an accumulation of colloid in the alveoli that the glands resembled simple colloid goitre, yet the Golgi apparatus of the glandular epithelium remained enlarged, indicating persistence of hypersecretion. Reversal of polarity of the Golgi apparatus was frequently observed. In one or two very grave cases of exophthalmic goitre Okkels¹⁵⁶ reported that 'some alveoli were found in which the number of cells with reversed Golgi apparatus amounted to 126 out of a total of 146'. He does not consider that the position of the apparatus in the cell bears any relation to the direction of secretory discharge. His views, however, closely approximate to those expressed by Ludford and Cramer when he says that 'there is no cytological or histological reason to assume that in exophthalmic goitre the thyrotoxicosis is due to anything else than an excessive hypersecretion into the blood-stream, preventing the accumulation of colloids'. Gillman⁶³ constructed models of thyroid cells in various phases of functional activity, and since his models demonstrated that the Golgi apparatus became extensively spread around the nucleus during the phase of active secretion, concluded that reversal of the apparatus does not occur and is only seen in single sections. He agrees that there is considerable enlargement of the apparatus in exophthalmic goitre, and that in this disease it gives 'incontrovertible evidence of the marked activity of the thyroid cell'. He confirms Okkels's observation that 'iodine administration in exophthalmic goitre does not diminish the activity of the thyroid cell, which, instead, maintains its marked secretory powers: the products of this thyroid activity are stored in the follicles in the form of colloid' (Gillman⁶⁴). Thomas¹⁸⁸ in his study of the human thyroid gland distinguished between cells which elaborate secretion and others, the function of which is to excrete the hormone into the blood-stream. He confirmed the observations of Cowdry, Ludford and Cramer, and Okkels, that the Golgi apparatus does sometimes become displaced towards the basal end of the cells. This, he states, occurs after a period of intense secretory activity. He denies, however, that the position of the apparatus bears any relation to the direction of secretory discharge.

Recently Welch and Broders¹⁹³ have reported the results of a cytological examination of seventy-three thyroid glands removed at

operations. Of thirty-five glands from patients with exophthalmic goitre, the Golgi apparatus was enlarged in all except one. This gland was removed from a patient who had been receiving iodine for 5 months before its removal. Further, in many of the follicles of glands removed from patients with exophthalmic goitre who had been treated with iodine, the cells showed marked regression, were flattened, and no enlargement of the Golgi apparatus was perceptible. Welch and Broders do not, therefore, agree with Okkels and Gillman as to the efficacy of iodine treatment as a means of inhibiting hyperfunctioning of the thyroid cells. Of the other glands they examined they found enlargement of the apparatus in nineteen of twenty-one glands removed from patients with adenomatous goitre with clinical symptoms of hyperthyroidism; and also in five of sixteen adenomatous goitres from patients who were not judged to be suffering from hyperthyroidism. From a critical estimation of clinical, histological, and cytological observations they concluded that 'appraising the size of the Golgi apparatus in the thyroid gland would therefore seem to offer a more delicate method of estimating cellular and glandular function than ordinary methods'.

iv. INTERACTION BETWEEN MICRO-ORGANISMS AND CELLS

a. *The Action of Bacteria on Cells.*

Our knowledge of cytological reactions to bacterial infections is very incomplete. No detailed cytological investigation has been made by modern techniques of the reactions of the cells of the principal organs in the case of any bacterial or protozoan infection. Most bacteria grow inter-cellularly in the body fluids, giving rise to toxins which have a generally injurious action, while others exert their action more particularly on specific tissues. There are various references in the literature to bacteria and bacterial toxins inducing degenerative changes in cells. Thus Ciaccio²² described the breaking up and vesiculation of mitochondria in cells of the convoluted tubules of the kidney following injections of diphtheria toxin and cultures of *Escherichia coli*; and Ciaccio and Scaglione²³ reported similar changes in cells of the choroid plexus following injections of *Brucella melitensis* and diphtheria toxin, and also in phthisis. Choja^{20, 21} studied the cytological changes in neurones of rabbits infected with certain viruses and some pathogenic bacteria and protozoa, and also poisoned by drugs such as strychnine, morphine, and nicotine. In general, the degenerative changes were of the same kind and involved reduction in the size of mitochondria, diminution in numbers, and finally their disappearance.

While the pathogenic bacteria are able to satisfy their nutritive requirements and grow in the body fluids, a few may also have an intra-cellular habitat. Thus *Neisseria intracellularis* and *Neisseria gonorrhoeae*

occur in large numbers in the cytoplasm of polymorphs, in which they have been reported to proliferate. According to Goodpasture⁶⁶ there is no evidence that the typhoid bacillus proliferates within the lumen of the gastro-intestinal tract, or within the blood-stream. He suggests that infection with this organism induces injury to the lymphoid tissue of the ileum, and results in the formation of plasma cells which become infected. Within such cells in lymphoid follicles of iliac and mesenteric lesions he found small Gram-negative bacilli, which he regards as 'an especially small bacillary form of *E. typhi* modified by their intracellular environment'. They occur in clusters often surrounded by a capsular material, suggestive of active intracellular proliferation. It is suggested that newly formed plasma cells constitute the cellular hosts of the bacilli affording nourishment and protection, not only during the period of incubation, but throughout the active course of the disease. Larger Gram-negative bacilli occur in the macrophages of the intestinal lesions, together with remains of phagocytosed lymphocytes, and in association with necrotic remains of macrophages from which they derive nourishment.

The most intimate association between living cells and bacteria occurs with certain of the Mycobacteria. These are relatively large acid-fast bacteria, and include the causative organisms of tuberculosis and leprosy in man and animals. When the tubercle bacillus becomes lodged in the tissues it brings about an accumulation of cells around it, within which it is said to proliferate. Such collections of cells, known as epithelioid cells, constitute tubercles—the characteristic lesions of the disease. As they increase in size by the addition of fresh cells, the centres undergo caseous degeneration owing to defective nutrition and accumulation of waste products of metabolism. The origin of the cells forming the tubercles has been the subject of much discussion, but the application of the tissue culture technique to this problem has done much to clarify it. Smyth¹⁸³ was the first to grow tissues *in vitro* with tubercle bacilli. He employed chick embryo tissues, and observed the accumulation of lymphocytes around the bacilli, and the formation of epithelioid cells. Their nuclei increased in numbers, and as the result of cell fusion multinucleate giant cells originated. More detailed cytological studies were published later by Maximow^{142, 143}, and Timofejewsky and Benewolenskaja^{186, 187}. Their investigations were carried out with rabbit tissues and blood leucocytes, and led to similar conclusions, namely, that epithelioid cells are transformed macrophages. Wermel¹⁸⁴ suggested they should be called 'epithelioid macrophages'. Morphologically, according to Lewis,⁹³ they are characterized by a central mass of fine granules stainable vitally with neutral red. This is surrounded by an intermediate zone of mitochondria amongst which fat droplets accumulate, especially in the cells at the centres of old tubercles. The oval

nucleus lies excentrically in this zone, adjacent to the central granular area, and contains a more clearly defined nucleolus than that of mononuclears. Peripherally there is a clear hyaline layer often extending out into thin membranous pseudopodia, like those of macrophages. In tubercles, Lewis recognizes all stages between monocytes, macrophages, and epithelioid cells. He considers that monocytes which phagocytose relatively large particles, such as erythrocytes, become macrophages, while those that accumulate small granules become epithelioid cells. Macrophages acquire epithelioid characters after their phagocytosed particles have been digested to fine granules: and under certain conditions epithelioid cells, by ingesting large particles, become typical macrophages. For Lewis, therefore, monocytes, macrophages, and epithelioid cells represent the various functional variations of the same cell type. It is to be observed that lymphocytes have no place in this scheme, and herein lies his disagreement with Maximow, Timofejewsky, and Benewolenskaja, who consider that lymphocytes are capable of transforming into polyblasts, and thence to macrophages. Other workers, however, have been unable to satisfy themselves that lymphocytes behave in this manner (Jonescu and Jonescu⁸⁰).

Multinucleate giant cells of the Langhans type originate from epithelioid cells either by their fusion, or by nuclear without cytoplasmic division, or by a combination of the two processes. Both have been observed by Lewis^{94, 95} in living cultures. These giant cells often attain considerable sizes with large numbers of nuclei. Lewis⁸⁹ figures one with forty-seven nuclei. The cytoplasm of these cells exhibits the same structure as that of the epithelioid cells, and the nuclei are usually arranged either crescentically or circularly around the margin of the central granular area.

There has been considerable discussion as to the relation between the epithelioid and giant cells, and the tubercle bacilli. Maximow^{142, 143} and Haagen speak of symbiosis between rabbit epithelioid cells and tubercle bacilli. Haagen⁷² reported that cultures of rabbit's lung with a moderate infection grow as well as non-infected controls. He described a chemiotactic action on the part of the bacilli for macrophages, but was unable to come to any conclusion as to whether the bacilli after phagocytosis were able to divide inside the epithelioid cells. Maximow considered that the presence of bacteria occasionally exerted a stimulatory action on rabbit epithelioid cells, and Timofejewsky and Benewolenskaja refer to mitosis occurring in cells which were crammed full with bacteria. The Jonescus also reported mitosis in epithelioid cells containing degenerate bacilli. Since man is more sensitive to tubercle bacilli than rabbits, Timofejewsky and Benewolenskaja infected cultures of human buffy coat and found that human leucocytes were far more sensitive to the action of the bacilli than were rabbit leucocytes. In heavily infected

cultures the cells rapidly degenerated: and in slightly infected cultures in which tubercles were formed degeneration occurred in 4 or 5 days. Most of the papers concerned with tuberculosis *in vitro* refer to the destruction of bacilli by macrophages and epithelioid cells under certain conditions. According to Wermel, if the cells are able to overcome the infection, the bacilli become surrounded by a vacuole, and are digested to a brown pigment. In his cultures of guinea-pig spleen he found that the bacilli had an inhibitory action upon growth; ultimately either the phagocytosed bacilli destroyed the cells, or else the cells digested the bacilli to pigment.

Although it has been said that 'tuberculosis is a disease of the monocytes', it is pertinent to note that masses of tubercle bacilli occur extracellularly in infected organisms; but since large numbers of bacilli occur in the cells of tubercles it is assumed that they find there a favourable habitat for their growth. The cellular changes occurring *in vitro* in the presence of tubercle bacilli, however, are by no means specific responses to infection with this organism. The formation of polyblasts, epithelioid cells, and multinucleate giant cells occurs regularly in cultures of lymphoid tissues. The same kind of cells are also formed by the monocytes of explanted tumours (Ludford¹²¹).

The leprosy bacillus is still more adapted to an intracellular mode of life than is the tubercle bacillus; and the former is rarely found free in the tissues. It induces the formation of similar nodular lesions composed of the epithelioid type of cells and giant cells. These cells become densely packed with bacilli. From such a focus of infection cells infiltrate the surrounding tissues. Growth is by accretion of fresh macrophages which become infected with the bacilli, rather than by mitosis which is rarely seen. Infection of the surrounding tissues appears to result from the breakdown of bacilli-laden cells. The result is that bacilli are found in many different kinds of tissue cells.)

Benewolenskaja⁷ investigated the reaction of cells to the leprosy bacillus in tissue cultures. He employed cultures of normal and leukaemic human blood, and also cultures of liver, spleen, and lungs of human embryos. The bacilli had no harmful action on the cells, and in appearance the infected cultures scarcely differed from the controls. The macrophages which migrated from the explants were very active in phagocytosing the bacilli. After 5 to 7 days macrophages became transformed into 'lepra cells'. These cells, also called 'foam', or Virchow cells, are sometimes multinucleate, and contain many bacilli. Their cytoplasm presents a foamy appearance owing to the presence of numerous droplets of a lipoidal nature. They soon degenerated *in vitro*. By the fusion of macrophages or epithelioid cells there originated multinucleate giant cells. The phagocytosed bacilli continued to proliferate intracellularly, and the so-called 'cigar packs'—groups of bacilli resembling

packets of cigars—were formed. Some of the phagocytosed organisms underwent degeneration, or were digested and gave rise to granules of yellow pigment.

Timofejewsky¹⁸⁵ had previously grown *in vitro* fragments of leprous tissue from skin nodules of lepers. He observed the outgrowth of macrophages containing the bacilli, and fibroblasts devoid of them. He described the development of lepra cells, and the formation of minute lepra nodules composed of epithelioid cells derived from macrophages. Mitoses were occasionally seen in cells containing numerous bacilli. That infected cells are not seriously injured is indicated by the observations of Cowdry³⁴. He found that their nuclei and mitochondria were normal in appearance: however, their functional activities were impaired. Their capacity to segregate trypan blue is reduced in rat leprosy; and they also fail to stain supravitaly with neutral red and Janus green. Cowdry also suggests that their nuclei contain relatively less thymonucleic acid than macrophages.

In the leprous lesions formed *in vivo* the bacilli form large globular masses called 'globi'. Cowdry³⁵ classifies them into three types: 'cigar packs', to which reference has already been made, are the smallest; 'seed globi', elongated bodies resembling oat seeds; and 'giant globi', which may attain a diameter of 150 μ or more. Evidence has been adduced that the latter develop intracellularly in giant cells.

It is of interest to note that leprosy bacilli, which have become adapted to an intracellular mode of life, resist attempts to cultivate them by the routine methods of bacteriological technique. Benewolenskaja⁷ reported their growth extracellularly in his degenerate cultures, and mentions that Timofejewsky's strain can be grown exclusively on clotted plasma, but grows much better in the presence of cells. It seems that the more micro-organisms become adapted to an intracellular habitat, the more difficult becomes their cultivation in artificial media. The *Rickettsiae* and viruses to be discussed in the following sections that have become so completely adapted to an intracellular mode of life are incapable of being cultivated apart from living cells, or in rare cases in association with dying ones.

b. *Rickettsiae*.

Rickettsiae have been defined by Cowdry²⁷ as 'Gram-negative, bacterium-like organisms of small size, usually less than half a micron in diameter, which are found intracellularly in arthropods, which may be more or less pleomorphic and stain rather lightly with anilin dyes, but which resemble in most of their properties the type species, *R. prowazeki*'—the causative agent of typhus fever. The generic name *Rickettsia* perpetuates the memory of Howard Taylor Ricketts, who died while investigating typhus fever; and the name of this species—

prowazeki—was given in memory of von Prowazek, who also forfeited his life in the same cause.

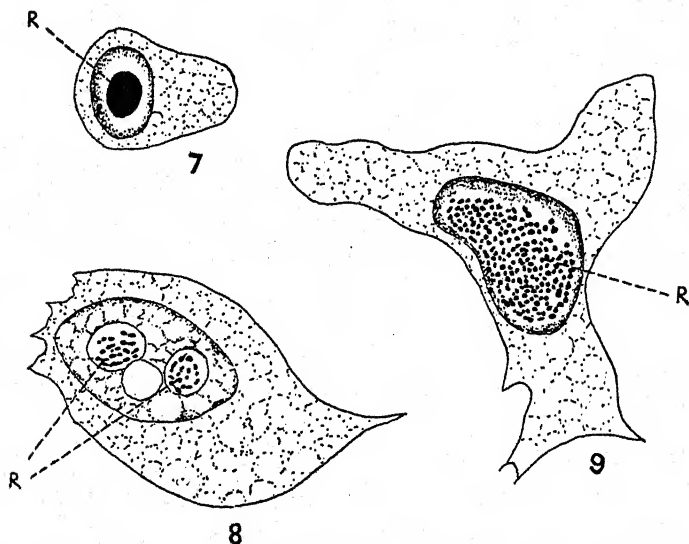
Rickettsiae are transmitted to mammals by the bites of insects and arachnids. In the arthropod vectors they occur both intercellularly and intracellularly; but in their mammalian hosts are almost entirely intracellular. They occur most frequently in endothelial cells, but most species have also been reported in other cells. They are the aetiological agents of typhus fever, trench fever, Rocky Mountain spotted fever, heart-water of cattle, and Tsutsugamuchi disease. The more typical *Rickettsiae* have not been grown in artificial media apart from living cells, in which respect they resemble viruses, but they are larger than the elementary bodies of viruses, and although there is conflict of opinion as to their ultrafiltrability, most evidence indicates that they can be retained by filters which allow the passage of viruses.

Rickettsiae exhibit a high degree of adaptation to an intracellular habitat in the bodies of mammals. According to Wolbach, Todd, and Palfrey¹⁹⁷ in man, *Rickettsiae* tend to form intracellular globular masses. This feature is even more pronounced with *R. ruminantium* in cattle suffering from heart-water. Cowdry²⁸ described colonies of the organism growing within vacuole-like cytoplasmic spaces filled with a clear fluid. The endothelial cells became considerably distended, but their nuclei seldom exhibited signs of injury. There was no evidence to indicate that the *Rickettsiae* stimulated cells to divide. Nicholson¹⁵³ found that in cells infected with the *Rickettsiae* of Rocky Mountain spotted fever—*Dermacentroxenus rickettsi*—the mitochondria were reduced in numbers, and those remaining lost their rod-shaped and filamentous form and became granular. This condition, as has been previously described, is characteristic of early degenerative cellular changes.

Cytological studies of *Rickettsiae* in tissue cultures emphasize the dependence of these organisms upon living cells for their growth and survival. Pinkerton and Hass^{164, 165} grew *in vitro* the membranous exudate of the testes of guinea-pigs infected with typhus. They obtained what they considered to be relatively pure cultures of mesothelium. *Rickettsiae* grew abundantly in these cells, and they exhibited considerable morphological variations—minute granules, diplobacilli, and chain-like forms. The best growths of the micro-organisms were obtained at 32° C. The majority of the cells then become distended with the *Rickettsiae*. They were found in cells in all stages of mitosis. It is of interest to note that 40 per cent. of the cells in mitosis were *Rickettsia*-free, while only 8.5 per cent. of cells not in mitosis were free of *Rickettsiae*. Mitosis was not sufficiently frequent to account for the infection of all the cells in the cultures. It seemed necessary to assume that the *Rickettsiae* were set free from some cells and gained entrance

to others. The same mode of transmission was suggested by Nishibe, Hosono, and Myazawa,¹⁵⁵ who worked with cultures of rabbit tissues infected with *R. orientalis* of Tsutsugamuchi disease. They observed no mitosis in infected cells.

In certain tissues of ticks Wolbach¹⁹⁶ found *Dermacentroxenus rickettsi* of Rocky Mountain spotted fever, massed within nuclei. They



TEXT-FIGS. 7-9. Intranuclear clusters of *Rickettsiae* of Rocky Mountain spotted fever in a tissue culture of scrotal sac exudate of a guinea-pig.

Fig. 7. Cluster of organisms with individuals not resolvable. Fig. 8. Early stage of infection. Fig. 9. Nucleus almost completely filled with *Rickettsiae*.

R, *Rickettsiae*. Semidiagrammatic, after Pinkerton and Hass¹⁶⁶.

have not been reported intranuclearly in infected cattle, but they have in tissue cultures of guinea-pig mesothelium. Pinkerton and Hass¹⁶⁶ grew the membranous exudate from guinea-pig testes infected with this *Rickettsia*. In several of their cultures the micro-organisms were seen only in the nuclei, while in others only the cytoplasm was infected. Both nuclear and cytoplasmic infection was rare. The *Rickettsiae* made their appearance in the nuclei as small spherical groups of closely packed organisms. Each cluster was surrounded by a definite halo. 'In some instances the individual organisms of a group could not be resolved, and the clusters appeared as hyaline masses' (Text-fig. 7). These are of particular interest owing to their resemblance to the intranuclear bodies characteristic of certain virus diseases to be discussed in the next section. At later stages of infection the clusters within a single nucleus fuse together, and the mass thus formed may come to occupy the greater part of the nucleus (cf. Text-figs. 8 and 9).

Rickettsiae do not survive long in degenerating cultures. According to Pinkerton and Hass¹⁶⁵, the *Rickettsia prowazeki* disappears in less than a week when the cells undergo degeneration. They remain visible only slightly longer than do the degenerating cytoplasm and nuclei of dying cells.

c. *The Action of Viruses on Cells.*

The study of viruses has opened up vistas of a world of minute organisms beyond the limits of resolution of visual microscopy. Our knowledge of their existence results from the diseases they cause in animals and plants. These include such infections as small-pox, rabies, influenza, yellow fever, and poliomyelitis (infantile paralysis) in man; foot and mouth disease in cattle; and the mosaic diseases of plants. That there exists an as yet unexplored realm of sub-microscopic non-pathogenic organisms seems highly probable.

Viruses have only been cultivated in the presence of living cells. They are described as ultra-microscopic and filter-passing organisms. The former attribute refers to their size. While the *Bacillus prodigiosum*, one of the smallest of bacteria, colonies of which are responsible for 'blood-spots' on damp bread, has a diameter of 0.75μ , the Psittacosis virus which is amongst the largest of the viruses is only one-third that size. Of the smallest, yellow fever virus has a diameter of about 0.022μ , and the viruses of poliomyelitis and foot and mouth disease are approximately 0.01μ in diameter, which is smaller than some of the largest organic molecules. It is obvious that particles of such sizes cannot be resolved by visual microscopy. Viruses are described as filter-passing, since they can be forced through filters which retain the smallest bacteria. Their actual sizes have been determined by three methods: by passage through Elford's collodion filters of known pore-size; by the technique of ultra-centrifugation; and in the case of some of the larger viruses by the direct measurement of ultra-violet photomicrographs (Barnard). The results obtained with the same virus by these different methods have been relatively uniform. The individual virus particles are usually known as 'elementary bodies'.

Many investigators have found it difficult to believe that the smallest of the viruses are living organisms. It seems scarcely feasible 'that there is room in a particle smaller than a molecule of haemocyanin and only two or three times the diameter of a haemoglobin molecule for the complexes necessary for organised life' (Laidlaw⁸³). Those who doubted the organismal nature of viruses found considerable support for their views in Stanley's discovery that from tobacco plants infected with mosaic disease there could be isolated a para-crystalline nucleoprotein which possessed all the properties of the virus (see Stanley¹⁸⁴). Since infection of plants with this purified protein leads to production

by the cells of more of the same virus, the suggestion has been advanced that viruses are complex auto-catalysts. Stanley suggests that 'the virus protein molecule may be able to cause smaller molecules already present in the cell, or smaller molecules produced by the cells as a result of the presence of the virus molecule to organize and combine to form a new virus molecule'. It is possible that all viruses are not of the same nature. Thus Rivers¹⁷² suggested 'some of them may be minute living organisms representing the midguts of the microbic world, others may be primitive forms of life unfamiliar to us, still others may be inanimate transmissible incitants of disease'. Laidlaw⁸³ advanced the suggestion that the diminution in size of the various viruses may be correlated with progressive loss of ferment-systems and auto-synthetic potentialities, and an increasing degree of dependence upon their cellular host. According to this conception, 'viruses are decadent forms of organisms degraded through long persistent parasitism', the smallest having attained 'the supreme summit of parasitism'.

Cells infected with viruses react in various ways. Andrewes¹ has emphasized that 'one can point to examples of every type of change produced in cells by the action of a virus, from pure necrosis to almost pure proliferation'. He cites, as instance of the former, foot and mouth disease. The filterable tumours of fowls afford examples of growth stimulation. With other viruses, such as those of fowl-pox and molluscum contagiosum of man, an initial cellular proliferation is followed by degeneration and necrosis.

Most viruses lead to the formation in infected cells of atypical bodies and granulations known as 'cell inclusions' or 'virus bodies'. They are formed, either within the nucleus, or in the cytoplasm, and with some viruses, such as small-pox, both types occur. The nature of these virus bodies has been the subject of much discussion. With most of them there has been controversy as to whether they represent accumulations or colonies of the viruses, or are simply reaction products of the cells. There has been an unfortunate tendency to study infected cells cytologically, without a preliminary study of the morphological changes which occur in the same cells during autolysis, in the absence of micro-organisms. At the beginning of this article we described the regressive changes that occur in cells, and the illustrations on Plate 1 demonstrate the variety of granules of various kinds which originate in cells grown under aseptic conditions, some of which, owing to their size, might easily be confused with viruses.

It would be impossible here to survey at all adequately the vast literature devoted to the action of viruses on cells, so we shall restrict our attention to a few viruses that induce the formation of characteristic inclusions. These are enumerated on the following page, together with their associated virus bodies.

Cytoplasmic inclusions

Fowl-pox	Bollinger bodies
Molluscum contagiosum of man	Molluscum bodies
Vaccinia (cow-pox)	Guarnieri-bodies
Rabies	Negri bodies
Mosaic diseases of plant	Granular and crystalline inclusions

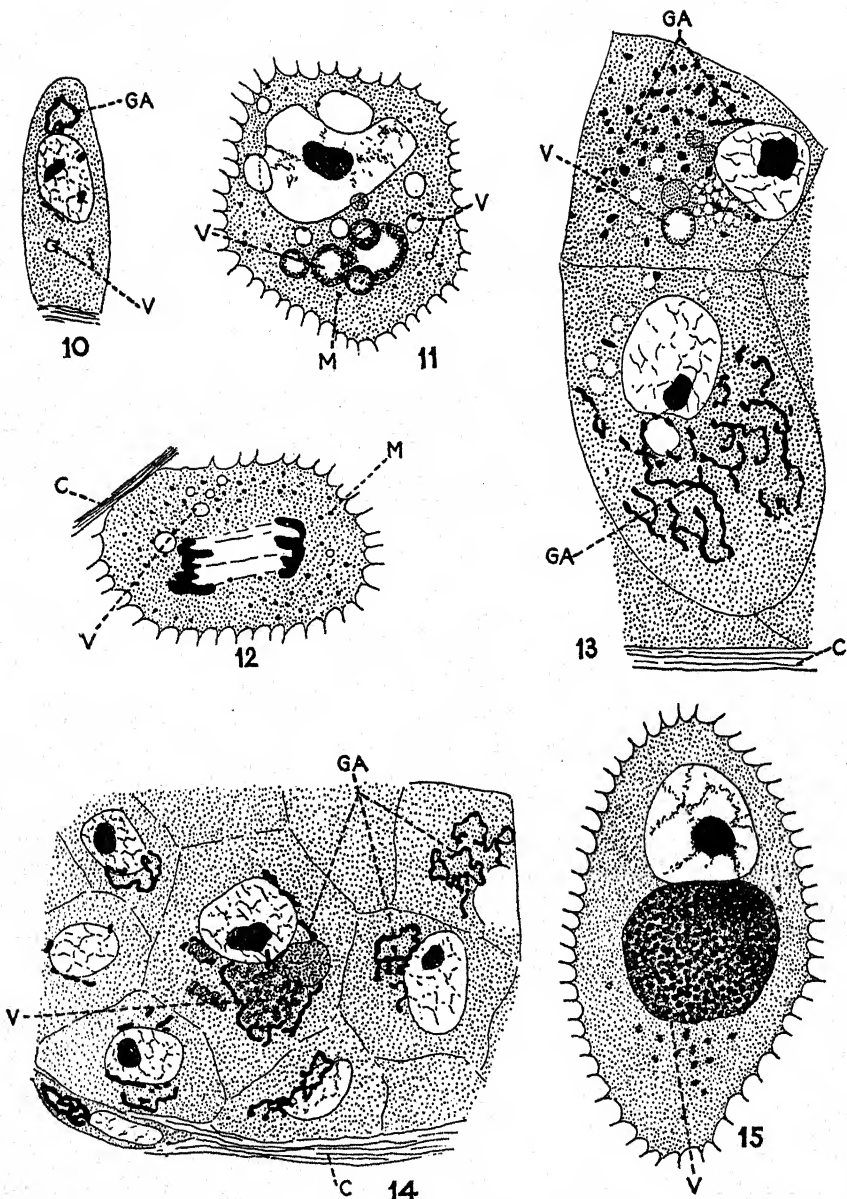
Intranuclear inclusions

Yellow fever	} Type A (Cowdry ³³)
Salivary gland disease of guinea-pigs and moles	
Virus III infection of rabbits	
Herpes	
Poliomyelitis (infantile paralysis). (Covell ²⁴ ; Hurst ⁷⁹)	} Type B
Borna disease of horses (enzootic encephalomyelitis)	

For full references to the literature the reader is referred to the reviews of Findlay and Ludford⁵⁴, Cowdry²⁹, Rivers¹⁷¹, Ludford¹¹⁷ and Stanley¹⁸⁴.

Cytoplasmic inclusions

Fowl-pox. Scarification of the skin of fowls with this virus induces the formation of wart-like nodules which often attain a considerable size. As the disease is contagious it is sometimes known as epithelioma contagiosum. The earliest indication of infection in the epidermal cells is the appearance of small cytoplasmic vacuoles. Text-fig. 10 shows such a cell impregnated to demonstrate the Golgi apparatus, occupying here its usual position at the apical pole of the nucleus. Many cells are seen in division containing these small vacuoles. One is shown in Text-fig. 12. The mitochondria here are of the typical granular form characteristic of mitosis in epidermal cells. In examining sections of lesions, as one passes from the lowest layers of the epidermal cells towards the surface of the skin, the cytological features exhibited are the expression of two processes—keratinization, and progressive infection of the cells with the virus. Keratinization alone is accompanied by a gradual disintegration of all the cell organs (Ludford¹⁰⁵), and it has been suggested that the formation of the large inclusions in this disease is correlated with disintegration of the cellular organization. In the course of the intracellular infection the cytoplasmic vacuoles increase in numbers and in size (Text-fig. 11) and appear ultimately to fuse, so that most cells come to contain a single large inclusion—the Bollinger body—occupying most of the cell. During their enlargement the vacuoles become surrounded by a lipoidal substance which blackens with osmic acid, stains with *Scharlach R* (Michaelis), and gives a positive reaction with Ciaccio's method for lipoids. After bleaching sections with hydrogen peroxide it is possible to demonstrate fine granules



TEXT-FIGS. 10-15. Reaction of epidermal cells of the chick to the virus of fowl-pox.

Fig. 10. Cell from lowest layer of stratum mucosum showing earliest indication of infection, small vacuoles in the cytoplasm. Fig. 11. Cell with virus inclusions, the larger with peripheral granules. Fig. 12. Telophase at early stage of infection, showing virus vacuoles and mitochondria. Fig. 13. Two cells at early stage of infection. The lower is considerably enlarged and its Golgi apparatus is hypertrophied and reversed in polarity; in the upper cell the Golgi apparatus is fragmented. Fig. 14. Group of cells showing altered polarity of the Golgi apparatus, and the formation of virus bodies in intimate relationship with the Golgi apparatus. Fig. 15. Cell with a single Bollinger body exhibiting a finely granular structure. C, connective tissue of dermis; GA, Golgi apparatus; M, mitochondria; V, virus bodies. After Ludford and Findlay¹³⁵.

within the larger vacuoles which have been regarded as the actual virus particles (elementary bodies), or accumulations of them (Text-fig. 15). During the early development of the virus bodies, cells become hypertrophied and the Golgi apparatus considerably enlarged (Text-figs. 13 and 14). Its normal polarity is lost and in some cells is completely reversed. A close topographical relationship between it and the developing virus bodies is often clearly apparent, and it seems probable that it may play some role in the formation or localization of the lipoidal substance which is deposited around the developing inclusions. It breaks up some time before the latter are fully formed (Ludford and Findlay¹³⁵). A similar hypertrophy of the Golgi apparatus followed by disintegration was described by del Rio-Hortega⁴⁴ in nerve cells in a case of paralytic rabies, and by Findlay^{52,53} in connective tissue cells in infectious myxomatosis of rabbits, and in some liver cells of animals infected with Rift Valley fever.

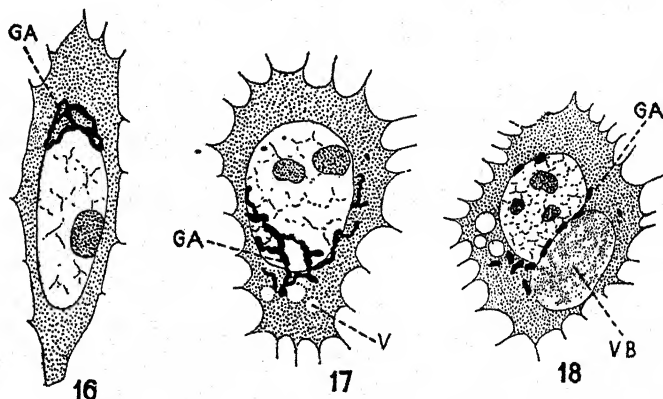
Fowl-pox inclusions were subjected to micro-incineration by Danks⁴³. They left a greyish-white residue consisting of minute particles of mineral ash, corresponding to the granules seen in suitably stained sections (Text-fig. 15). He suggested that these granules might not themselves be the virus particles, but that they served as loci for its adsorption. Woodruff and Goodpasture¹⁹⁸ demonstrated conclusively that Bollinger bodies contained the virus by isolating single bodies by means of the micro-manipulation technique. One of these after washing in saline and inoculation into the skin of a fowl would produce a typical fowl-pox lesion, while the saline in which it was suspended before inoculation was innocuous.

Molluscum contagiosum of man. According to Goodpasture and King⁶⁷, molluscum bodies are very similar to the Bollinger bodies. They differ from the latter in the absence of a lipoidal envelope. After tryptic digestion they are sticky and gelatinous (Goodpasture and Woodruff⁶⁸). The fully formed body according to Van Rooyen¹⁹⁰ is pear-shaped with a semi-translucent 'cap' at its conical extremity. This is the thinnest part of the body wall, and the most easily ruptured with liberation of the included granules. Van Rooyen considers this virus body resembles in miniature a fungus sporangium. Tests with Lugol's iodine indicate that its outer covering is composed of carbohydrate. After micro-incineration this is burnt off, leaving a granular residue derived from its contents (Van Rooyen¹⁹¹).

Vaccinia (Cow-pox). The Guarnieri-bodies produced by this virus have been the subject of much investigation, and most conflicting views have been expressed as to their nature. Since they are formed in epidermal cells in which keratinization occurs, with extrusion of nucleolar material into the cytoplasm, there has been some confusion of this with the virus inclusions. Most recent work favours the view that

Guarnieri-bodies represent a developmental phase in the life cycle of the virus. Bland and Robinow⁹, who observed the results of infecting cells in tissue cultures with the virus, came to the conclusion that the Guarnieri-bodies represent colonies of elementary bodies enveloped in a matrix.

In epidermal cells of the rat cornea infected with vaccinia, and treated cytologically for the demonstration of the Golgi apparatus, minute



TEXT-FIGS. 16-18. Reaction of rat corneal cells to vaccinia virus (cow-pox).

Fig. 16. Normal cell with Golgi apparatus at apical pole of nucleus. Fig. 17. Enlargement and reversal in polarity of the Golgi apparatus in association with virus vacuoles. Fig. 18. Fragmented Golgi apparatus and large Guarnieri-body.

GA, Golgi apparatus; V, virus vacuole; VB, virus body (Guarnieri-body). After Ludford¹¹².

vacuoles are seen in the cytoplasm. The Golgi apparatus of such cells is frequently enlarged and often exhibits complete reversal of polarity. This condition is illustrated in Text-fig. 17, which should be compared with Text-fig. 16 showing the form and position of the apparatus in a normal cell. Other cells contain larger vacuoles that are seen to contain extremely fine granules which are interpreted as accumulations of the elementary bodies. Such a cell is represented in Text-fig. 18. It will be observed that the Golgi apparatus is fragmented, and this therefore would indicate a later stage of infection (Ludford¹¹²). The large vacuolar inclusions appear to be formed as the result of the enlargement and fusion of the smaller vacuoles. Ultimately cells become distended with large vacuoles filled with fine granules. That Guarnieri-bodies develop from elementary bodies which penetrate cells, become surrounded by vacuoles inside of which they proliferate, and finally fuse to form a virus colony included within a fluid matrix was the conclusion to which Eisenberg-Merling⁴⁹ also came as the result of the study of living infected cells of the rabbit cornea by dark-ground illumination. It is of

interest to note that the enlargement of the Golgi apparatus, its reversal of polarity, and topographical relationship with the developing inclusions is closely paralleled with the cytological process described previously in cells infected with fowl-pox.

Psittacosis is another virus which forms cytoplasmic bodies of a well defined character, composed of virus particles embedded in a matrix. They have been studied *in vivo* and *in vitro* by Bedson and Bland^{4,5}, and Bland and Canti⁸.

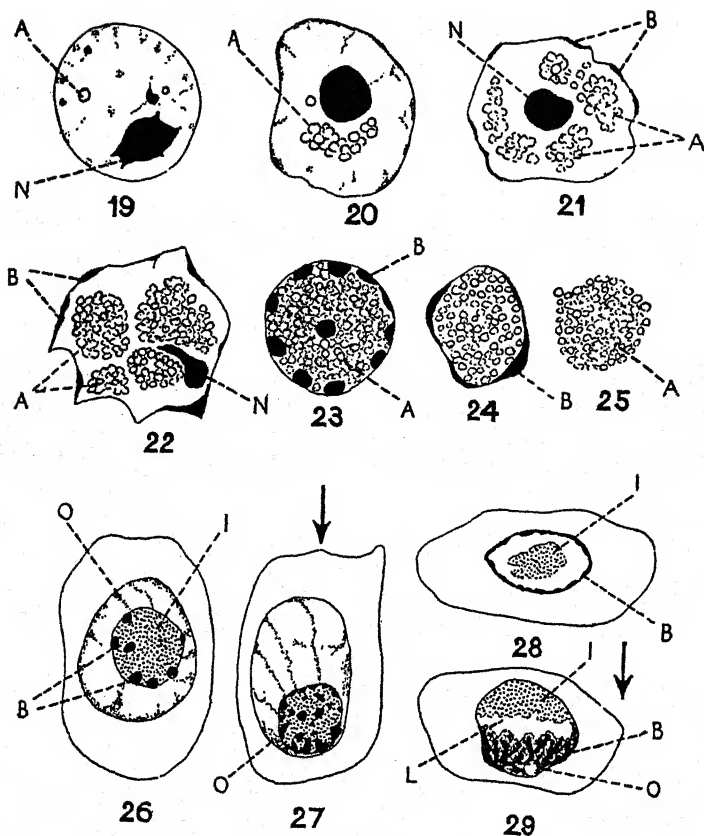
Rabies. Two types of inclusions have been described in the nerve cells of animals and man infected with rabies: small hyaline granules, called 'lyssa bodies', and the larger Negri bodies, which consist of a ground substance containing a central body surrounded by chromatoid granules. The latter are sufficiently distinctive to be of diagnostic value. There is considerable difference of opinion as to whether they represent colonies of elementary bodies or are simply products of cellular disintegration. The latter view is supported by recent workers (see Covell and Danks²⁶).

Virus Diseases of Plants. Cell inclusions are formed in some virus diseases of plants, but not in others. Two types have been described, amorphous granular bodies, and crystalline ones. Both contain virus together with a second constituent differing in the two types. Sheffield¹⁸¹ has suggested that viruses may be rendered insoluble in the cytoplasm by precipitation in the form of such complexes, and that the formation of these inclusions may represent an intracellular protective mechanism.

Intranuclear Inclusions.

Cowdry²³ distinguishes between two types of intranuclear inclusions in virus diseases, which he designates types A and B. Those of type A are formed by the accumulation of granular acidophil material, accompanied by the displacement on to the nuclear membrane of the basophilic nuclear component. The nucleoli are altered and displaced peripherally where they disintegrate, and the inclusion-laden nuclei ultimately become disrupted. Type B inclusions are usually spherical, often hyaline droplet-like bodies, arising in nuclei which otherwise appear normal. Margination of the basophilic nuclear substance does not occur in the same way, but it is displaced peripherally if the inclusion becomes very large.

Luger and Lauda¹³⁶ interpreted these inclusions as arising from what they called 'oxychromatic' degeneration. The process of formation of a characteristic intranuclear inclusion is shown in Text-figs. 19-25, reproduced semi-diagrammatically after Cowdry and Kitchen³⁶. They illustrate the formation of the inclusion bodies of yellow fever in the nuclei of liver cells of the monkey, *Macacus rhesus*, demonstrating stages in the progressive accumulation of acidophil granules, and the deposition of basophilic substance on the nuclear membrane. The intranuclear



TEXT-FIGS. 19-29. Intranuclear virus bodies.

Figs. 19-25. Yellow fever inclusions in liver cells of *Macacus rhesus* monkeys. (Semi-diagrammatic, after Cowdry and Kitchen³⁶.) Figs. 19-22. Stages in the development of the acidophil particles: and in the margination of its basichromatin. Figs. 23-4. Late terminal stage. Fig. 25. Nuclear membrane has disappeared leaving a mass of acidophil granules. A, acidophil granules; B, basichromatin; N, nucleolus.

Figs. 26-9. After Lucas¹⁰¹. Fig. 26. Duct cell of submaxillary gland of the ground mole containing intranuclear inclusion of maxillary gland disease. Fig. 27. Similar cell after ultracentrifugation for an hour at approximately 500,000 times gravity. Fig. 28. Corneal epithelial cell of rabbit containing virus body of herpes. Fig. 29. Similar cell after ultracentrifugation. B, basichromatin; I, inclusion body; L, nucleoplasm; O, oxychromatin.

inclusions of liver cells in Rift Valley fever arise in a similar manner according to Findlay⁵³. Intra-hepatic inoculation of herpes virus into the liver of the monkey, *Cebus hypoleucus* is followed by a different mode of formation of inclusion bodies. Cowdry and Kitchen³⁶ describe them as being formed from 'more or less even cloud-like deposits of acidophil particles in the centres of the affected nuclei', also the separation between the acidophilic and basophilic constituents of the nucleus

is more marked in herpes than in yellow fever. Other differences have been described between the various intranuclear inclusions. Thus the herpetic inclusions are more finely granular and less compact than those of the virus III inclusions of rabbits (Cowdry³¹). The development of the large inclusions within hypertrophied cells in sub-maxillary gland disease of guinea-pigs and moles is not accompanied by margination of basophilic material. The latter remains in association with the virus body and is responsible for its tendency to basophilic staining. Lucas¹⁰¹ has sought to correlate the degree of association between basichromatin and virus bodies with the cellular specificity of the viruses. 'When a virus is very selective, as it is in the submaxillary viruses, generally there is found a corresponding compatibility between inclusion body and the chromatin of the infected cell. In contrast, viruses having low specificity, such as herpes, which is cosmopolitan in infective potentialities, show low compatibility with the nuclear material.' Lucas demonstrated this difference between the viruses he cites by subjecting cells containing the two types of virus bodies to ultracentrifugation. The nucleus of an epithelial cell of the rabbit containing a central virus body and marginal basichromatin, as shown in Text-fig. 28, became stratified after centrifugation, as seen in Text-fig. 29. Basi- and oxychromatin were displaced centrifugally; the inclusion body centripetally. The virus body of a sub-maxillary gland cell of the mole (Text-fig. 26), was, on the other hand, displaced by centrifugation together with the basic chromatin in the direction of the centrifugal force, and strands of oxychromatin remained stretched between it and the nuclear membrane (Text-fig. 27).

The application of histochemical tests to the intracellular inclusions of yellow fever, herpes, chicken-pox, sub-maxillary disease, virus III, and Rift Valley fever—have demonstrated that:

- (i) they do not stain with Sudan III or blacken with osmic acid;
- (ii) they are negative to the Bensley-Macallum test for masked iron;
- (iii) they fail to stain with the Feulgen reaction for thymonucleic acid, except in some cases very faintly.

When subjected to micro-incineration the mature virus bodies unlike nucleoli leave very little or no ash (Cowdry and Kitchen³⁶; Cowdry³²; Rector and Rector¹⁶⁹; Findlay⁵³; Horning and Findlay⁷⁶).

Acidophil intranuclear inclusions resembling those produced by viruses have been reported in cells under such conditions that there is no reason to assume any relationship with a virus infection—in kidney cells of many mammals and birds (Cowdry, Lucas, and Fox³⁷) and in tissue cultures of foetal leptomeninges (Fischmann and Russell⁵⁶). Other inclusions have been found which are so like those of known virus aetiology that they have led their discoverers to assume they have been produced by unknown viruses. Examples of these are the inclusions found in liver

cells of dogs, in salivary gland cells of *Cebus fatuellus*, and in kidney cells of *Macacus rhesus* by Cowdry and Scott^{39,40,41}; in kidney cells of sewer rats by Hindle⁷⁴; and the inclusions described by Hass⁷³ in liver and adrenal cortex cells of an infant afflicted with a fatal disease characterized by hepato-adrenal necrosis. Intranuclear inclusion bodies resembling to varying degrees those produced by known viruses have been induced artificially in various nerve cells by injections of large quantities of distilled water and by hypertonic solutions of glucose, NaCl, and NaHCO₃; also in mononuclears and giant cells by certain aluminium and ferric compounds, and by carbon (Olitsky and Harford¹⁵⁸). In the latter case attempts to demonstrate a virus were unsuccessful. Furthermore, Belt⁶ found intranuclear inclusions resembling those of yellow fever in liver cells in cases of liver necrosis following severe burns.

From the examples which have been cited it seems clear that the presence of intranuclear inclusions alone should not be accepted as proof of virus action. Whether any of the nuclear inclusions caused by viruses represent actual accumulations of virus particles is disputable. In justification to those who favour the view that certain of the inclusions consist of elementary bodies embedded in an acidophil matrix (see Markham¹⁴¹) it is pertinent to recall that intranuclear accumulations of *Derma-centroxenus rickettsi* occasionally appear identical with intranuclear virus bodies (see p. 242).

V. CYTOLOGY OF CANCER

Malignant cells are characterized by their capacity for unlimited uncontrolled proliferation. Cells of any of the tissues of the body may become malignant and form a cellular mass which invades the surrounding tissues. Small clusters of cells may be swept away in blood-vessels or lymphatics and give rise to secondary growths, or metastases on becoming lodged in other organs. Thus cancerous growths of the skin of mice induced by painting with tar or carcinogenic hydrocarbons frequently metastasize in the axillary lymph nodes and lungs. A malignant growth originating in pigment cells of the human eye (melanotic sarcoma) may become so dispersed that most of the organs ultimately contain secondary growths if the patient survives long enough.

Malignant growths derived from epithelial cells are known as carcinomata, and those arising from connective tissue cells as sarcomata. When grown as tissue cultures a typical carcinoma forms a sheet of closely adherent cells (Plate 2, Fig. 1), while a typical sarcoma tends to grow as a cluster of spindle-shaped cells (Plate 2, Fig. 2), but there are many exceptions to this generalization.

It is customary to speak of tumours as being either filterable or non-filterable. A filterable tumour, after crushing to a pulp and mixing with

saline, yields after Berkefeld-filtration a cell-free filtrate, which after injection into an animal of the same species reproduces the same type of progressively growing tumour as that from which the extract was prepared. Filterable tumours can also be transmitted by desiccated tissue, and from fragments of tumour preserved in 50 per cent. glycerine. Non-filterable tumours cannot be transmitted by any of these methods, but only by cellular grafts.

Rous¹⁷⁵ was the first to demonstrate that malignant growths could be transmitted by cell-free filtrates. He worked with five filterable sarcomata of fowls of different histological types, and subsequently many others have been discovered (see Foulds⁵⁸). The potency of filtrates prepared from these tumours varies from time to time. Thus Gye and Andrewes⁷¹ reported that for a period of 6 months the Rous sarcoma No. 1 failed to yield active filtrates, but did so spontaneously at the end of that period. The properties of the active agents contained in filtrates are essentially similar to those of viruses in general, so that they are usually known as tumour viruses. We are completely ignorant as to the means whereby these, or other viruses, stimulate cell proliferation. With regard to the tumour viruses two possibilities have been suggested. Either the virus alters a normal cell in some way so as to induce a kind of mutation and then merely proliferates as a passive passenger in the presence of the actively growing malignant cells, or the virus becomes intimately linked with the cellular mechanism so as to form some sort of cell-virus complex, which is the cause of malignant growth. Unequivocal evidence as to which is the correct explanation is lacking, but from what is known of other viruses and their relation to cells the latter seems the more feasible interpretation. Of all the known viruses, the tumour viruses exhibit the highest degree of cellular specificity, and adaptation to their cellular hosts (Ludford¹³⁰).

Acidophil intranuclear inclusions similar to those characteristic of other virus diseases have been described by Lucké¹⁰³ in cells of a filterable tumour of the frog kidney, discovered by him. From his figures they appear to conform to the specifications of Cowdry's type A inclusions. Somewhat similar intranuclear inclusions have been described by Turevich¹⁸⁹ in Fischer's filterable fowl sarcoma, which is indistinguishable from the Rous sarcoma No. 1.

Amongst the non-filterable tumours are included the greater number of tumours of laboratory animals which have not been transmitted by cell-free extracts. Nevertheless, viruses have been shown to be concerned in the origin of certain of this class. Shope¹⁸² discovered that a virus was responsible for inducing wart-like growths—papillomata in cotton-tail rabbits. The virus could be transmitted to domestic rabbits, in which papillomata were formed that became malignant and gave rise to secondary growths in lymph glands and lungs (Rous and Beard¹⁷⁶);

but these malignant tumours were not filterable, although immunological evidence has been adduced of the persistence of virus in them. Rous and Kidd¹⁷⁷ showed that if tar was applied to the skin of rabbits so as to induce a local overgrowth of the epidermis (hyperplasia) and the Shope virus was then injected intravenously, malignant growths very soon developed at the site of the skin lesions. In considering the significance of these tumours we are once more faced with the problem as to whether the papilloma virus alone, or the combination of virus and tar, merely bring about a constitutional alteration of some kind in normal cells which is the basis of malignancy, or whether the virus enters into an intimate relationship with the cells and thereby stimulates them to continuous proliferation.

There remain for consideration the vast majority of cancers of experimental animals and man, of spontaneous origin or induced by chemical and physical agents, in the genesis of which no virus has been found to be concerned. Tumours have been induced experimentally by such diverse agents as cysticercus larvae, aniline, tar, a variety of hydrocarbons, dyestuffs, ultraviolet light, and X-rays and radium. Apart from their capacity to induce malignancy, these agents have only one cytological action in common. They all bring about aberrations of mitosis. Since ultraviolet light and X-radiations, which are carcinogenic, also induce mutations in germ cells, it has been suggested that these, as well as other carcinogenic agents, induce mutations in somatic cells, and that malignant cells are one of the types of mutations produced. The normal chromosome number is by no means uncommon in malignant cells, so that it is postulated that cancer-producing mutations would be of the type involving changes in the distinctive gene material, which is probably of a protein nature. This conception of the mechanism of malignancy would afford an explanation of the retention of specific cytological characters of different strains of transplantable tumours, which experience indicates are retained indefinitely. Attention has been frequently drawn to the similarities between viruses and genes (Astbury²; Gowen⁶⁹), and it is not impossible that the intimate union of tumour viruses with cells might bring about the same end result in metabolism as the type of gene mutation envisaged. It should be pointed out that attempts to produce mutations in *Drosophila* by means of carcinogenic hydrocarbons have been unsuccessful (Auerbach³), although by their application to cultures of *Paramoecium*, abnormal strains of this organism have been produced (Mottram¹⁴⁷).

While it is conceivable, but unproved, that non-filterable as well as filterable tumours might have a common virus aetiology, there is justification on general biological grounds for believing that the aberration of cellular functioning which is responsible for malignancy might be brought about, either by the addition of an extrinsic agent, or by some

permanent alteration in the intracellular mechanism which is transmissible at mitosis. Many examples could be cited of the same cytological effect being induced by both biological and chemical agents. Normally, segmentation of the ovum is initiated by the sperm, but the numerous researches on parthenogenesis have demonstrated by what a variety of different methods the same result can be attained, even in the higher mammals. Specially significant in this respect are the similar cytological effects induced by viruses and chemicals. We have referred previously to the formation of acidophil intranuclear inclusion associated with virus diseases, such as those of yellow fever and herpes, and the production of identical bodies by Olitsky and Harford¹⁵⁸, when no virus could be detected. The inclusions produced by chemical agents gave the same histochemical reactions as those resulting from virus action.

Apart from the occurrence of acidophil intranuclear inclusions, characteristic of virus diseases, in some filterable tumours, the cytological features presented by malignant cells are essentially similar whether or not tumours are filterable (Ludford¹³³). No specific morphological criterion of malignant cells has been discovered, but it would be erroneous to conclude that there are no cytological differences between malignant cells and their non-malignant prototypes. Cancer cells are not simply cells which have acquired rapidity of growth. They are specifically altered cells. The transplantation of tumours has demonstrated that the specific cytological characters of tumours are retained over prolonged periods of time. Some strains of transplantable tumours have been propagated for more than thirty years, and during that time retained unaltered their characteristic features—general rate of growth, degree of differentiation, and morphological structure. Further, these are retained just the same whether the tumours are propagated by transplantation in animals, or grown as tissue cultures. The growth of normal and malignant tissues in cultures for long periods has demonstrated most definitely the essential difference between rapidly growing normal cells and malignant ones. Chick fibroblasts after cultivation *in vitro*, for a period far in excess of the normal life span of fowls, cease to grow on inoculation into chicks (Carrel¹³), while cultures of the Ehrlich mouse carcinoma, after twelve years' cultivation *in vitro*, when inoculated into mice continued to grow and form tumours of the same type as that from which they were originally explanted (Fischer and Davidsohn⁵⁵).

Malignant cells often exhibit a high degree of dedifferentiation. In general the more differentiated the cells the slower is the rate of growth. There are strains of transplantable tumours of the mammary gland the cells of which exhibit secretory activity, and others in which it has never been observed. Melanotic sarcoma cells elaborate pigment, and while some sarcomata induce considerable fibril formation, others do not.

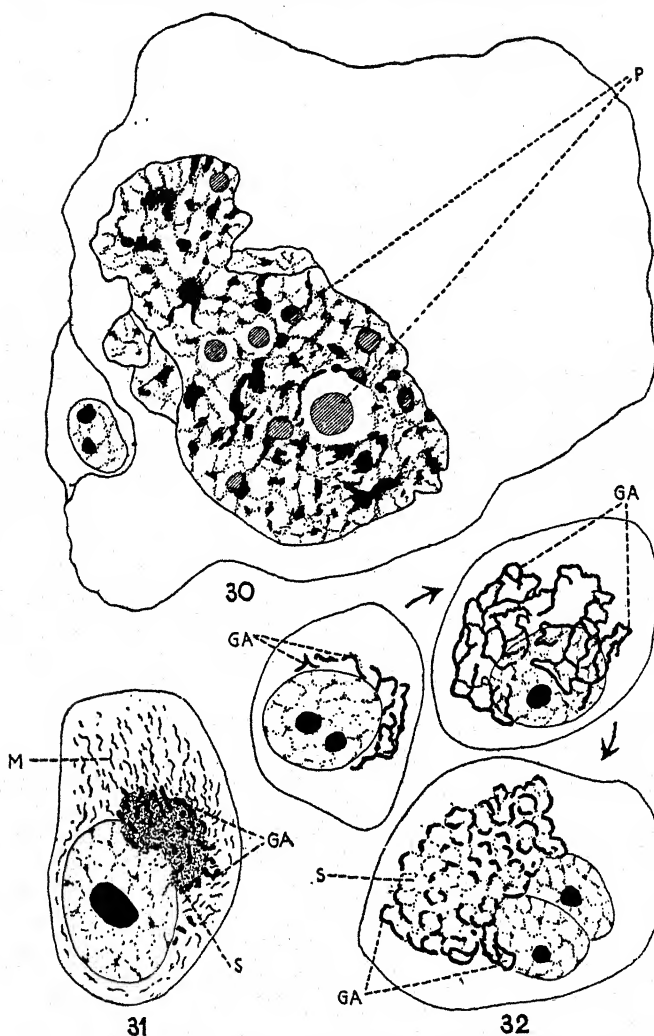
Malignant liver cells may excrete bile, and various tumours of the endocrine glands have been reported, the cells of which secrete to varying extents their specific hormones. Such cases render it improbable that cellular dedifferentiation can be an essential factor in carcinogenesis, as some have maintained.

Malignant cells exhibit the widest range of morphological abnormalities. Amongst cells of the same tumour there is frequently a considerable variation in size, as is shown in Text-fig. 30, which represents two cells from a melanotic sarcoma of the horse. Enlargement of cells is accompanied by increase in number of mitochondria, and the Golgi bodies become larger and more numerous. The polarized disposition of the cytoplasmic organs characteristic of so many normal cells is usually retained by malignant cells adjacent to well formed stromata, but in the absence of the latter polarity is invariably lost (Ludford^{106, 108}).

The structure of the Golgi apparatus of malignant cells corresponds to that of their prototypes or to stages in their dedifferentiation (Ludford¹⁰⁹). The specific secretions of malignant gland cells are formed in relationship with the Golgi apparatus in the same manner as has been described in most glands. Text-fig. 32 illustrates the formation of milk in human mammary carcinoma cells, and Text-fig. 31 abortive secretory activity in a mouse mammary carcinoma.

Areas of necrosis are common in tumours and seem to result from deficiencies in their blood-supply. Consequently, the cells of tumours exhibit all kinds of degenerative changes. Hypertrophied nuclei may become extensively lobed, and nuclear budding and fragmentation are common. Cell fusion (plasmogamy) results in syncytial masses being formed. Nuclear and cytoplasmic organs exhibit the regressive changes which were described at the beginning of this section.

Since the early studies of Pianese and von Hanseman the mitotic aberrations of malignant cells have received considerable attention. Although most of the abnormalities which have been reported in malignant cells have been produced experimentally in non-malignant cells, yet the occurrence of so many in cancerous tissues is a distinctive feature (Ludford^{115, 116}). An experienced cytologist would have no difficulty in distinguishing between a tissue culture of actively growing embryonic cells and one of malignant cells on this basis alone. Almost all the types of mitotic abnormalities conceivable are met with in malignant cells (Plate 3). There is great variation in the numbers of the chromosomes, and the latter vary in shape from small coccoid-bodies to long filaments; sometimes they are fragmented. The spindle is often multipolar, but occasionally absent altogether (Ludford¹¹⁴). The chromosomes may fail to form a complete metaphase plate, and often some remain apart without attachment to the spindle. Unequal distribution of chromosomes to daughter cells results from delayed separa-



TEXT-FIGS. 30-2. Variation in size, and examples of functional activity in malignant cells.

Fig. 30. Small cell and a greatly hypertrophied cell from a melanotic sarcoma of the horse. P, intranuclear pigment granules. After Ludford¹⁰⁴. Fig. 31. Mouse mammary carcinoma cell, showing relation between Golgi bodies and secretion. After Ludford¹¹⁹. Fig. 32. Human mammary carcinoma cells showing relation between Golgi bodies and milk. Original. GA, Golgi apparatus; M, mitochondria; S, secretion.

tion of chromatids, or failure of some to separate at all. Then nuclear division is not always followed by cytoplasmic division. The occurrence of abnormal mitoses is more frequent in some tumours than in others. The fact that the abnormalities are retained during growth *in vitro*

implies that they are an inherent property of malignant cells and not the result of the actions of toxic metabolites accumulated *in vivo* owing to the deficient vascularity of tumours, as was at one time suggested. It would be extremely interesting to know whether any malignant growths occur without atypical mitoses of any kind.

The completion of mitosis in the normal manner is conditioned by an orderly series of changes in the viscosity of different regions of the dividing cell. It seems probable that toxic substances which cause mitotic abnormalities do so by bringing about alterations in the colloidal state of the cytoplasm and chromosomes (Ludford¹²⁹). Hence the suggestion that the mitotic abnormalities of tumour cells are not determined by nuclear, but by cytoplasmic factors. Corroborative evidence has been forthcoming from the study of the cytoplasm and nucleoplasm of living malignant cells in tissue cultures. That the cytoplasm of sarcoma cells differs morphologically from that of fibroblasts has been reported by Lewis and Gey⁹⁸, Lewis⁹⁶, Carrel and Ebeling^{15,16}, and Ludford¹²⁵. A comprehensive study of the living cells of numerous sarcomata was made by Lewis⁹⁷. He says:

'Each type of malignant cell usually has one or more characteristics which differentiate them from normal ones. Some of the common differences which distinguish the malignant cells are increase in size of cell and nucleus, increase in density of cytoplasm, increase in number and decrease in size of the mitochondria, increase in the amount of nucleolar material, increase in thickness of the nuclear membrane and the granular condition of the nucleoplasm.'

The differences between the cytoplasm and nucleoplasm of fibroblasts and sarcoma cells are best seen by examining living cells by dark ground illumination. These are illustrated in Plate 4, which shows a typical fibroblast (Fig. 1) and cells of the Jensen rat sarcoma (Figs. 2 and 4), and of the Crocker sarcoma (Fig. 3). The more granular nature of the cytoplasm of the sarcoma cells is obvious.

Although Chambers and Ludford^{17,18} were unable to detect any differences in the viscosity of normal and malignant cells by the micurgical technique, or in the intracellular hydrogen ion concentration by the injection of colour indicators, the behaviour of tumour cells *in vitro* suggest that the colloidal state of their cytoplasm differs in some way from that of normal cells (Ludford^{123,124}). Fibroblasts are able to grow and spread out on a glass surface in a fluid culture medium such as serum. Many strains of tumours are quite unable to do so under normal conditions of growth *in vitro*; but there is much variation in the behaviour of the cells of different strains of tumours in this respect (Ludford¹³¹). The rounding up of cells of mouse sarcoma No. 37 in serum, and their spreading out in a plasma clot is illustrated in Plate 5. Fig. 1 is enlarged one and a half times more than Fig. 2. Similar differences in behaviour have been reported between epithelial cells and carcinoma cells.

Probably related to a difference in the ultra-structure of their cytoplasm is the inability of malignant cells to segregate acid vital dyestuffs such as trypan red and trypan blue in the same manner as their normal prototypes. Fibroblasts segregate these dyes as granules in association with vacuoles. No sarcoma has yet been found, the cells of which segregate these dyes *in vivo* as do fibroblasts (Ludford¹¹³; Foulds⁵⁷). This is the more remarkable as Duran Reynals⁴⁷ has pointed out that an abnormal amount of dyestuff collects in tumours *in vivo*. In tissue cultures, probably owing to the altered condition of cells spread out in plasma clots, some segregation of acid vital dyes by sarcoma cells occasionally occurs; but there is a clear difference in the manner in which the dyestuff is segregated by such cells and normal fibroblasts. While milk in secreting mammary gland cells is stained by trypan blue, and the same cells when not engaged in secretory activity segregate the dyestuff in granular form, malignant mammary gland cells take up very little or no dyestuff whether they be of the differentiated secretory type or have lost all secretory capacity (Ludford¹²⁰).

It is not known whether the failure of malignant cells to segregate acid vital dyes like their normal prototypes is the result of some difference in the ultra structure of their cytoplasm preventing the actual segregation of the dyestuff in granular form from its semi-colloidal solution, or whether it results from a less permeable plasma membrane. Lipoid soluble basic dyestuff and coloured compounds of the Sudan III type readily penetrate living malignant cells, and in the case of the former colour cytoplasmic vacuoles, while the latter stain fat droplets (Ludford¹²⁶); but these are cases of the staining of preformed structures and differ essentially from the segregation of dyestuffs as granules, which are new cytoplasmic formations containing a protein constituent (Chlopin¹⁹). Further investigations on the colloidal state of the protoplasm of malignant cells are required to elucidate the significance of the observations which have been recorded.

vi. CONCLUSION

In the preceding pages we have discussed in a necessarily superficial manner some of the more important of the pathological aspects of cytology. The attempt has been made to give some idea as to how cells react to adverse conditions, to demonstrate the cellular mechanism tuned to its highest pitch in functional hyperactivity, and to trace the interaction between micro-organisms and cells which, on the one hand, results in cell destruction, and on the other as the result of an increasing dependence of the micro-organism upon its cellular host leads to a progressive perfecting of a symbiotic relationship, favouring the survival of both at the expense of the organism as a whole. The climax is reached with the filterable tumours where the union between virus and cell is so

complete as to confer upon the cell new characters and the remarkable property of unlimited proliferation. The non-filterable tumours remain the final enigma. Do they represent the complete loss of identity of viruses incorporated completely in the larger and more complex mechanism of the cell?

The problems encountered in this survey have been many and varied, the gaps in our knowledge extensive, so that at the present time a synthetic discussion seems unprofitable. We are left with the conception of the cell as a functioning unit, with its microscopically resolvable organellae reacting to abnormal conditions as parts of a unified system. Only the grossest parts of the mechanism are visible under the microscope, which conveys but the crudest idea of the underlying ultra-microscopic complexity. By its exploration we may reasonably hope to acquire a more fundamental insight into the mechanism of cellular functioning under normal and pathological conditions.



FIG. 1. Accumulation of degenerating mitochondria around the nucleus.



FIG. 2. Ill-defined mitochondria and degeneration granules accumulated at the side of the nucleus, and surrounded by fat droplets.

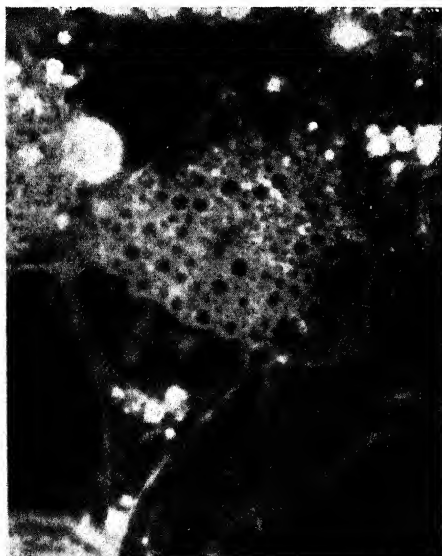


FIG. 3. Vacuolation of the cytoplasm.
(Original.)

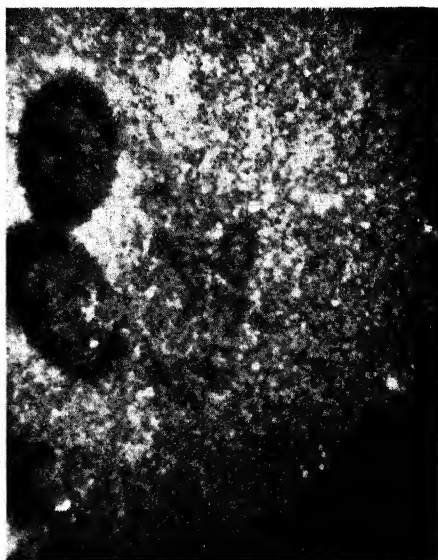


FIG. 4. Binucleate cell filled with degeneration granules in the midst of which is a reticulate space resembling the Golgi apparatus.

Degenerative changes in fibroblasts in tissue cultures.
Magnification approximately 1,200. Figs. 1, 2, and 4 after Ludford¹²⁸.

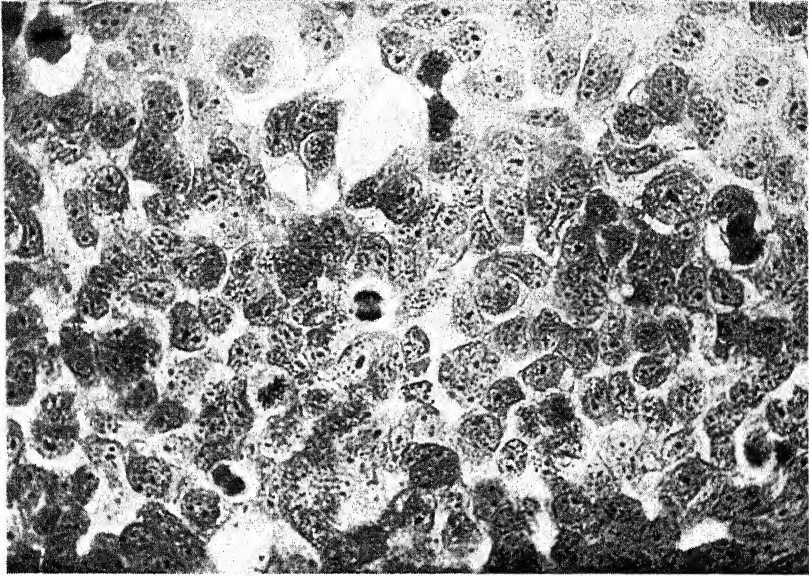


FIG. 1. Mouse carcinoma⁶³ of the Imperial Cancer Res. Fd. $\times 450$. (After Ludford¹²⁴.)



FIG. 2. Jensen rat sarcoma. $\times 450$. (After Ludford¹²⁵.)

Characteristic type of growth of tissue culture of a carcinoma and a sarcoma.

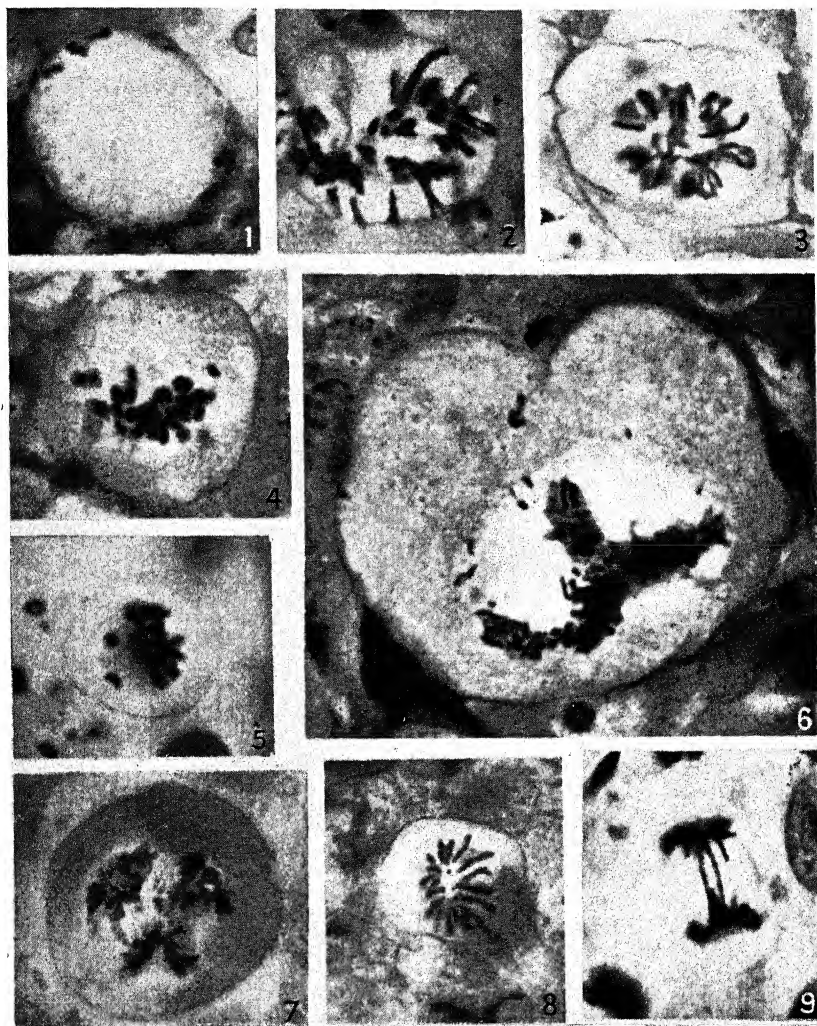


FIG. 1. Chromosome formation without spindle development in Jensen rat sarcoma cell: chromosomes displaced to cell periphery.

FIG. 2. Similar aberration in mouse sarcoma 37, but photomicrograph shows surface of cell instead of section as in Fig. 1.

FIG. 3. Metaphase in cell of Fibiger's tar tumour: same magnification as cell of the same tumour shown in Fig. 6.

FIG. 4. Chromosomes reduced to granular form in mouse carcinoma 63.

FIG. 5. Incomplete metaphase plate with two aberrant chromosomes in mouse carcinoma 63. (Original.)

FIG. 6. Mitosis in giant cell of Fibiger's tar tumour. More than tetraploid number of chromosomes, some fragmented: deformed multipolar spindle.

FIG. 7. Multipolar mitosis in Jensen rat sarcoma growing *in vitro*.

FIG. 8. Haploid number of chromosomes in mouse carcinoma 206.

FIG. 9. Delayed separation of chromosomes at telophase in mouse carcinoma 63. (Original.)

Figs. 1 and 2 after Ludford¹¹⁴, Figs. 3, 4, 6, and 8 after Ludford¹¹⁶, and Fig. 7 after Ludford¹²⁵.

Aberrations of mitosis in malignant cells.

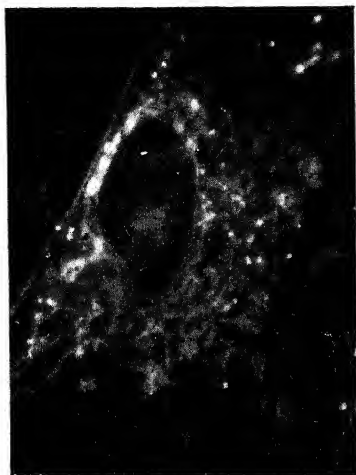


FIG. 1. Well-spread rat fibroblast with large filamentous mitochondria. (Original.)

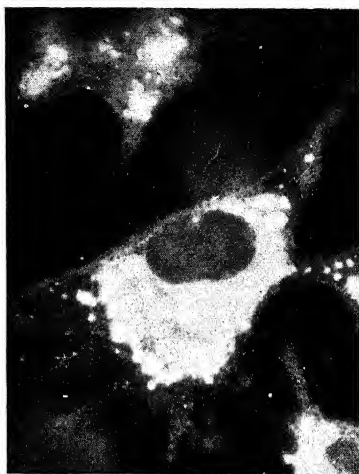


FIG. 2. Jensen rat sarcoma cell.

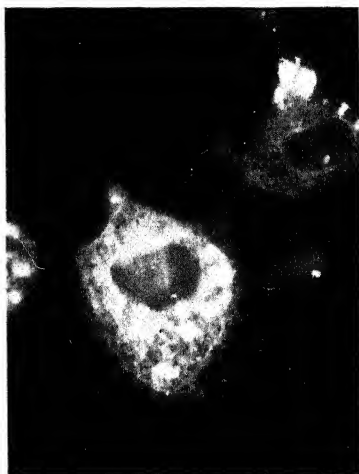


FIG. 3. Crocker mouse sarcoma cell.

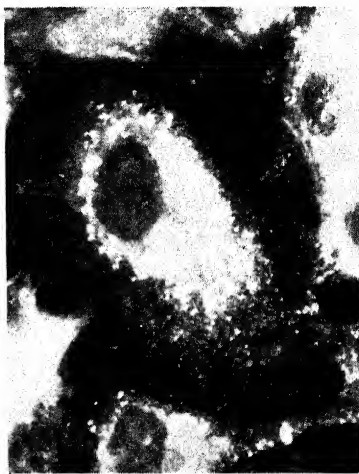


FIG. 4. Jensen rat sarcoma cell more spread out than in Fig. 2. (After Ludford¹²⁵).

Photomicrographs by dark-ground illumination of a normal fibroblast and of malignant fibroblasts (sarcoma cells) showing the more granular character of the latter, and their finer mitochondria.

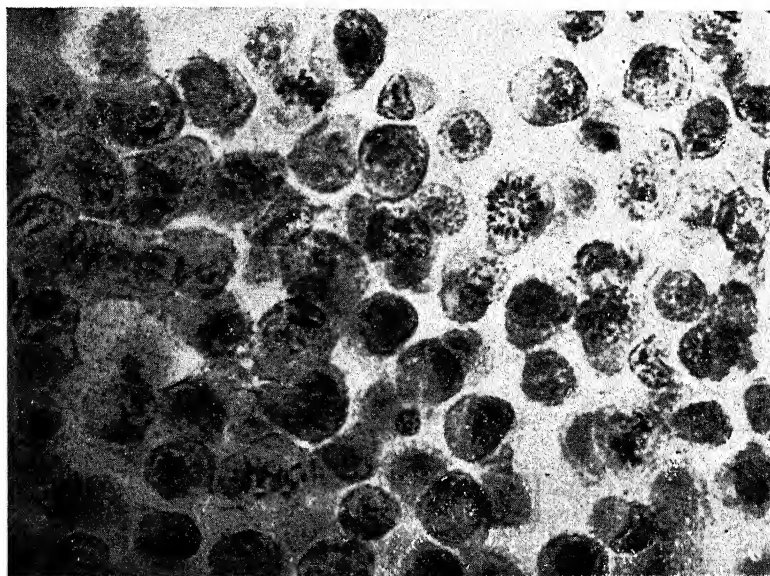


FIG. 1. Rounded cells in serum. $\times 670$. (After Ludford¹²⁵.)

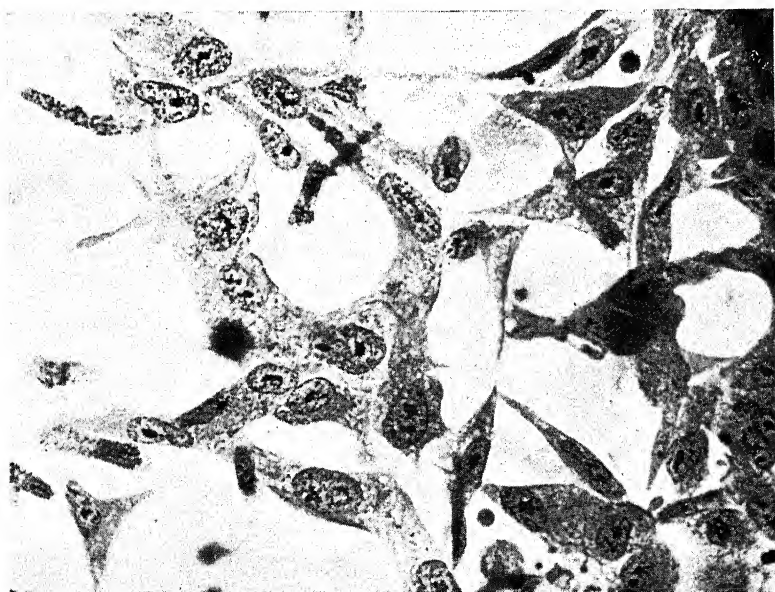


FIG. 2. Well-spread cells growing in plasma clot. $\times 450$. (Original.)

Different behaviour of the cells of a sarcoma (mouse sarcoma No. 37)
explanted in serum and in clotted plasma.

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